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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

SHOZO SAKUMA, ET AL. : EXAMINER: A.S. WEHBE

SERIAL NO: 09/548,290 :

FILED: APRIL 12, 2000 : GROUP ART UNIT: 1632

FOR: ANIMAL MODEL FOR ALLERGIC DISORDERS

DECLARATION UNDER 37 C.F.R. § 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VA 22313-1450

SIR:

Now comes Dr. Shozo Sakuma who deposes and states that:

1. I am a graduate of Kyoto university and received my pharmaceutical doctor's degree in the year 2002.

2. I have been employed by Fujisawa Pharmaceutical Co, Ltd. for 24 years as a scientist in the field of Pharmacology and Toxicology.

3. I am an inventor in the above-identified application.

4. I have read and am familiar with the claims of the above-identified application, a copy of which is attached hereto as Exhibit 1.

5. I have read and am familiar with the following publications: (1) Morita et al., Journal of Dermatological Science, 19, 37-43 (1999), (2) Yasue et al., Cellular Immunology 181, 30-37 (1997) and (3) Gad, Toxicology 93, 33-46 (1994). Copies of Morita et al., Yasue et al. and Gad are attached hereto as Exhibits 2, 3 and 4, respectively.

6. Hiroi et al., Jpn. J. Pharmacol. 76, 175-183 (1998), a copy of which is attached hereto as Exhibit 5, describe using conventional NC/Nga mice to examine the effect of FK506 and steroid ointment on the development of spontaneous dermatitis. FK506 ointment (Protopic®), which is efficacious for atopic dermatitis, shows significant inhibition of spontaneous dermatitis in the NC/Nga mouse. On the other hand, commercially available betametasone valate ointment, a steroid ointment which is generally known for treating patients with atopic dermatitis, showed only a marginal effect on the development of dermatitis. See Figure 2, Figure 3 and lines 6-10 of the Abstract.

In contrast, Sasakawa et al., Int Arch Allergy Immunol 2004; 133: 55-63 (hereinafter referred to as "Sasakawa et al. I"), a copy of which is attached hereto as Exhibit 6, report that both FK506 ointment and Rinderon®-V, which is the trademark for the betametasone valate ointment mentioned above, remarkably suppressed dermatitis in the mouse claimed in the above-identified application (see Figure 1).

The mice used by Sasakawa et al. I fall within the scope of Claim 29 of the above-identified application. Sasakawa et al. state at page 56, column 2, that the mice used in the study described therein were prepared according to the procedure described in Sasakawa et al., Int Arch Allergy Immunol 2001; 126: 239-247 (hereinafter referred to as "Sasakawa et al. II"), a copy of which is attached hereto as Exhibit 7. The mice produced in Sasakawa et al. II fall within the scope of Claim 29 of the above-identified application. That this is so is demonstrated by the fact that NC/Nga mice were treated with mite extract on the ears under specific pathogen free conditions, so that the mice display symptoms of atopic dermatitis. See, for example, the Abstract.

The following table summarizes the situation with respect to treating atopic dermatitis.

	Literature Reference	Steroid Ointment	FK506 Ointment
Human Patient		O	O
Conventional NC/Nga mouse	Hiroi et al.	X	O
SPF NC/Nga mouse	Sasakawa et al. II	O	O

O: Effective

X: Ineffective

As shown in the table above, the mouse claimed in the above-identified application, i.e., the "SPF NC/Nga mouse," can actually estimate the efficacy of Rinderon®-V, which is sold as a drug for treating atopic dermatitis. The mice described by Hiroi et al., which, as shown above, cannot be used to estimate the efficacy of Rinderon®-V, are similar to the mice described by Morita et al. These results suggest that the mouse claimed in the above-identified application is more suitable for evaluating drug efficacy in patients with atopic dermatitis as compared to the mouse described by Morita et al.

7. Moreover, the mouse claimed in the above-identified application shows symptoms of atopic dermatitis more quickly as compared to the mouse described by Morita et al.

Sasakawa et al. II reports that the ear thickness was definitely increased from day 5-8 and the ear thickness and the severity of the skin lesions were increased with further mite antigens injections until day 18 (Figure 1 and page 242, column 1, lines 2-5). This means that the mouse claimed in the above-identified application can be used for the screening of a new drug for atopic dermatitis only after one to three weeks from the commencement of the mite antigen injection.

In contrast, the mouse described by Morita et al. would require at least 4 weeks from the commencement of applying the mite before screening could begin. Morita et al. state at page 39, under "Results," as follows:

> Four week-old mice: NC/kuj, BALB/c and C57BL/6, were kept together with mite-infested NC mice for 2 weeks to receive mites and then separated. Desquamation and crust due to scratch of skin became remarkable in NC/Kuj at 8 weeks of age.

Thus, the mouse claimed in the above-identified application is more suitable as a model mouse in the screening of new drug for atopic dermatitis than the mouse described by Morita et al. This is because the mouse claimed in the above-identified application requires a shorter amount of time for the test as compared to the mouse described by Morita et al.

8. The superior properties of the mouse claimed in the above-identified application over the mouse described by Morita et al. discussed above are unexpected from the combination of the references cited by the Examiner.

Morita et al. describe the use of fur mites to induce atopic dermatitis in NC mice. The mites were not treated with the mites under a specific pathogen free environment.

Yasue et al. describe a study designed to evaluate the hyposensitizing activity of recombinant Der f 2 (rDer f 2). See the Abstract. In the study, mice were cosensitized with rDer f 2 crude and mite extract, and then challenged with crude mite extract (experiment 2, page 30, second column, first paragraph under the section entitled "Materials and Methods"; see also the section entitled "Experiment 2" at page 32, first column). Thus, in the procedure described by Yasue et al. the crude mite extract is administered by inhalation via an aerosol (see page 32, first column, bottom). As described at page 33, column 2, in the paragraph preceding the "Discussion" section, "inhalation of mite extract provoked late-phase airway inflammation characterized by neutrophil influx in the mice." Yasue et al. provide a detailed discussion of the biochemical responses provoked in the study described in that reference

(Experiment 2, see page 33, second column, bottom to page 36). Significant by its absence is any mention of skin lesions, atopic dermatitis, or IgE.

The Gad publication is a review article describing the mouse ear swelling test (known as "MEST"). This publication provides the current version of the MEST protocol (as of 1994, when the review was published). See the Abstract. Gad fails to discuss using MEST to evaluate the effect of mite extract. Gad is also silent about using MEST to prepare a mouse model for atopic dermatitis.

One with those references in hand would not have been able to predict that the mouse claimed in the above-identified would have the properties discussed in paragraphs (6) and (7) above. There is simply no suggestion that the mouse claimed in the above-identified application would have those properties from the combination of Morita et al., Yasue et al. and Gad.

9. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

10. Further deponent saith not.

Shozo Sakuma

Shozo Sakuma

2004.12.8.

Date

EXHIBIT 1

29. A mouse suitable as a model for atopic dermatitis, wherein the mouse is a NC/Nga mouse which has been sensitized with a mite extract on the ear(s) under a specific pathogen free environment, such that the animal displays at least one symptom of atopic dermatitis caused by the mite extract.

30. The mouse of Claim 29, wherein said symptom is skin lesions on the ear(s) of the mouse.

32. The mouse of Claim 30, wherein the skin lesions are at least one member selected from the group consisting of erythema, edema, excoriation, and scaling.

33. The mouse of Claim 30, wherein the skin lesions are erythema.

34. The mouse of Claim 29, wherein the symptom is ear swelling.

35. A method of producing the mouse of Claim 29, comprising:

maintaining a NC/Nga mouse in a Specific Pathogen Free environment and sensitizing the ear(s) of the mouse with the mite extract.

36. The method of Claim 35, wherein the animal is sensitized with the mite extract for at least 5 days.

37 A method of screening for an agent for effectiveness against atopic dermatitis, comprising:

applying at least one agent to the mouse of Claim 29,

determining whether the agent reduced one or more symptoms of atopic dermatitis,

correlating a reduction in said one or more symptoms with effectiveness against atopic dermatitis, and

correlating a lack of reduction in said one or more symptoms with ineffectiveness against atopic dermatitis.

EXHIBIT 1

29. A mouse suitable as a model for atopic dermatitis, wherein the mouse is a NC/Nga mouse which has been sensitized with a mite extract on the ear(s) under a specific pathogen free environment, such that the animal displays at least one symptom of atopic dermatitis caused by the mite extract.

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applying at least one agent to the mouse of Claim 29,

determining whether the agent reduced one or more symptoms of atopic dermatitis,

correlating a reduction in said one or more symptoms with effectiveness against atopic dermatitis, and

correlating a lack of reduction in said one or more symptoms with ineffectiveness against atopic dermatitis.

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Fur mites induce dermatitis associated with IgE hyperproduction in an inbred strain of mice, NC/Kuj

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Abstract

An inbred strain of mice, NC, has been introduced as an animal model for atopic dermatitis because the mice develop dermatitis associated with severe scratch preceded by elevated serum IgE level when kept in conventional conditions. Although hypersensitivity to some environmental factors is suggested to cause dermatitis, the precise factor remains unclear. As the mice maintained under conventional conditions were often infected with fur mites, we investigated whether an infection of fur mites induces skin lesions in NC. Infection with the fur mites induced NC to develop skin lesions associated with highly elevated serum IgE, whereas no obvious skin lesions were observed in BALB/c and C57BL/6, and the elevation of serum IgE level was minimal in these two strains of mice. The role of the fur mites in the manifestation of skin lesions and IgE hyperproduction was confirmed by eliminating the fur mites by treatment with ivermectin. In addition, the existence of specific IgE antibody to *Myocoptes musculinus* antigen in the sera of mite-infested NC was detected by the antigen-induced histamine release from bone marrow-derived cultured mast cells after sensitization with the serum. These results suggest that continuous exposure to fur mite antigen is a potential factor in the development of dermatitis in NC. We provide a new model system of antigen-induced dermatitis for investigating the role of IgE in eliciting dermatitis. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: NC mouse; IgE; *Myocoptes musculinus*; Atopic dermatitis; Ivermectin

1. Introduction

An inbred strain of mice, NC, has been known to develop dermatitis associated with severe scratch when the mice are kept under conven-

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tional conditions, whereas the mice remain healthy when bred in clean conditions [1]. Manifestation of the skin lesion of NC follows an increase in the level of serum IgE. The dermatitis developed in NC, thus, is considered to be a model for human atopic dermatitis [1]. Hypersensitivity to some agents existing in conventional conditions is suggested to induce dermatitis in NC [1], but the precise factor is still unknown. Mice are often infested with fur mites such as, *Myobia musculi* or *Myocoptes musculinus*, in conventional conditions. Although these mites occasionally cause production of a specific IgE to the mite antigen in some strains of mice, the mites are usually harmless [2]. The aim of this study was to investigate a role of fur mite infection in the manifestation of skin lesions and the IgE hyperproduction in NC.

2. Materials and methods

2.1. Animals

NC/Kuj is a substrain of the NC strain which has been derived from Japanese fancy mice and was established as an inbred strain in 1955 [3]. The original NC strain was transferred to the Institute of Medical Science, University of Tokyo, and after being renamed as NC/Jms the breeding was continued. In 1979 the strain (NC/Jms) was transferred to the Institute for Experimental Animals, Faculty of Medicine, Kanazawa University, where the infested fur mites were eradicated and the strain was renamed as NC/Kuj. Specific pathogen-free (SPF)-NC/Kuj were kindly provided by Dr J. Hayakawa (Institute for Experimental Animals, Faculty of Medicine, Kanazawa University), and bred in Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine. BALB/c and C57BL/6 were purchased from Japan SLC (Hamamatsu, Japan).

2.2. Infection protocol of fur mites

Four-week-old mice NC/Kuj, BALB/c or C57BL/6) were kept together with fur mite (*Myocoptes musculinus*)-infested NC mice for 2 week in isolated clean rooms with air filtration an thereafter separated. In some experiments NC Kuj were treated with a spray of ivermecti (Merck, Sharp & Dohme BV, Holland) [4,5]. Iver mectin was diluted 1:50 (v/v) in 50% propylen glycol solution in aqua (final concentration of 0. mg/ml) and each mouse received three bursts o spray from a distance of approximately 30 cm The spray was repeated three times with 1-weel intervals. Fur mites and their eggs were observe under a microscope by applying Scotch tape o the skin lesions.

2.3. Histological examination

Skin was carefully excised from mice anaes thetized with diethylether. Each biopsy specime was fixed with 5% formalin neutral buffer solutio and was paraffin embedded. Sections of 5 μn. thickness were cut for staining with either haema toxylin–eosin or toluidine blue.

2.4. Determination of serum total IgE

The concentration of serum total IgE was de termined by using mouse IgE EIA kit (Yamas Shoyu, Chiba, Japan).

2.5. Determination of serum antigen-specific IgE

Collection of mites and mite extract was pre pared as described elsewhere [2]. Briefly, mic heavily infested with *Myocoptes musculinus* wer killed by cervical dislocation, placed in a tray a 4°C for 1 h at room temperature. The fur mite were collected by brushing their hairs, homoge nized and proteins were extracted with 10 m phosphate-buffered saline. The protein concentra tion of the mite antigen preparation was 95 μg/m as determined by UV absorption at 280 nm. *Der matophagoides farinae* (Df) antigen and *Candid albicans* antigen were kindly provided by Tori Pharm. Corp. (Tokyo, Japan).

Bone marrow-derived cultured mast cell (BMMC) were obtained by culturing bone mar row cells from 6-week-old male NC/Kuj as previ ously described [6]. Suspensions of BMMC were

incubated with mouse serum (final concentration with 5%) at 37°C overnight, washed twice with medium, and then 2×10^5 cells were dispensed in 1.5-ml polypropylene test tubes containing various concentrations of antigens, and further incubated at 37°C for 30 min. After centrifugation ($150 \times g$ for 5 min at 4°C), supernatants were mixed with the same volume of 0.5 N perchloric acid. Cell pellets in the test tubes were resolved in 1 ml of 0.25 N perchloric acid to release histamine.

Histamine content in samples was determined by using an automated histamine analyser (Tosoh, Osaka, Japan), and percentage histamine release was calculated by the formula:

$$\frac{\text{histamine in supernatant}}{(\text{histamine in pellet} + \text{histamine in supernatant})} \times 100$$

2.6. Statistical analysis

Differences between groups were analyzed by using the Student's t-test. Probability (P) values less than 0.05 were considered to be significant.

3. Results

Four-week-old mice: NC/Kuj, BALB/c and C57BL/6, were kept together with mite-infested NC mice for 2 weeks to receive mites and then separated. Desquamation and crust due to scratch of the skin became remarkable in NC/Kuj at 8 weeks of age, and the skin lesions continued progressively thereafter. However, no obvious skin lesion was observed in BALB/c and C57BL/6, although mites were detected in all of the mice. At 14 weeks of age, serum IgE levels in NC were above 20000 ng/ml, whereas those in BALB/c and C57BL/6 remained at low levels (Fig. 1). Mice kept without mite infection (SPF-NC) in clean rooms showed no remarkable skin lesions. Serum IgE levels of mite-infested NC increased markedly, whereas those of SPF-NC remained in a low level (Fig. 2). No appreciable difference was observed between the male group and the female group

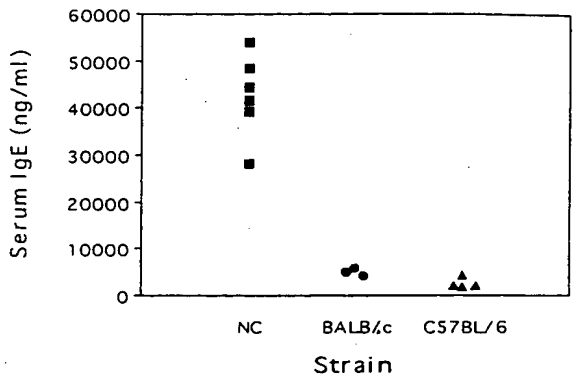

Fig. 1. Serum IgE levels in the three strains of mice, NC/Kuj, BALB/c and C57BL/6. Four-week-old mice were infected with *Myocoptes musculinus*, and their serum IgE levels were measured 10 weeks after mite infection. The IgE level of 4-week-old mice was below the detection limit (below 100 ng/ml) in all strains examined.

To investigate the pathology of skin lesions, we took skin specimens from mice 8 weeks after fur mite infection, and compared with those from SPF-NC at the same age. Haematoxylin–eosin staining of the biopsy specimen revealed acanthosis and an increased number of dermal infiltrates of mononuclear cells and polymorphonuclear leukocytes (mostly neutrophils and occasionally eosinophils) in mite-infested NC, whereas no sig-

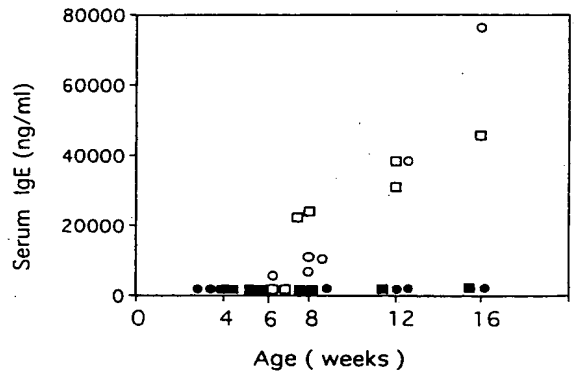

Fig. 2. Serum IgE levels in the mite-infected NC and SPF-NC. Sera were collected from (■) SPF-male NC, (●) SPF-female NC, (□) mite-infested male NC and (○) mite-infested female NC.

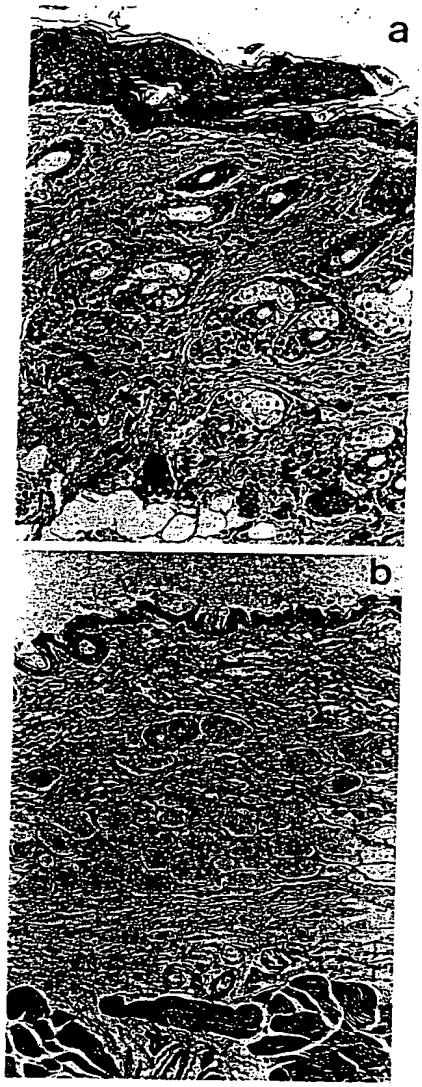

Fig. 3. Photomicrographs of skin biopsies taken from sites of NC/Kuj mice (haematoxylin and eosin, × 100). (a) Eight weeks after mite infection, (b) an SPF-NC at the same age.

nificant change was seen in SPF-NC (Fig. 3). Toluidine blue stain of the specimen showed an increased number of mast cells in the upper dermis compared with those from SPF-NC (data not shown).

Four-week-old NC/Kuj which had been bo from mite-infested parent mice were divided in two groups; one treated with ivermectin spray a the other treated with vehicle alone. After 8 wee ivermectin-treated mice showed no skin lesio and mites were not detected in these mice exce two having empty egg cases, whereas vehicl treated mice showed marked skin lesions wit 100% mite infection (Table 1). Serum IgE leve remained at low levels in the ivermectin-treate mice, whereas those in the vehicle-treated mic increased significantly ($P < 0.001$ (Fig. 4). At 1 weeks of age, mean serum IgE levels were 405 ± 455 ng/ml (mean ± S.D., $n = 7$) in ivermectin treated NC and 53371 ± 29383 ng/m (mean ± S.D., $n = 7$) in vehicle-treated NC.

Ten-week-old mice showing skin lesions whic had been infested with mites were divided int two groups; one treated with ivermectin spray, th other treated with vehicle alone. Determination o serum IgE levels revealed no significant differenc between these two groups before treatment. Fou weeks later the IgE levels increased markedly i the vehicle-treated group, whereas that in th ivermectin-treated group decreased. Eight week later the difference of serum IgE levels becam significant between these two groups ($P < 0.001$); mean serum IgE levels were 57870 ± 26462 ng/m (mean ± S.D., $n = 7$) in vehicle-treated NC and 9766 ± 3072 ng/ml (mean ± S.D., $n = 6$) in ivermectin-treated NC (Fig. 5). Skin lesions of the ivermectin-treated mice markedly improved, whereas those of the vehicle-treated mice remained unchanged.

Specific IgE to fur mite antigen was determined in the sera of mite-infested NC by using the antigen-induced histamine release from BMMC. When BMMC were incubated with sera obtained from mite-infested NC, the BMMC released more than 70% of total histamine in stimulation with fur mite antigen, whereas the BMMC incubated with SPF-NC sera failed to release histamine (Fig. 6). The BMMC that were sensitized with mite-infested NC sera also released approximately 20% of total histamine in response to 10 μg/ml Df antigen, but not *Candida* antigen (up to 10 μg/ml).

Table 1
Efficacy of ivermectin treatment in eliminating fur mites in NC mice

Group	4 weeks (before treatment)			12 weeks		
	Mites	Eggs	Skin lesions	Mites	Eggs	Skin lesions
Ivermectin-treated	7/7[a]	7/7	0/7	0/7	2/7	0/7
Vehicle-treated	7/7	7/7	0/7	7/7	7/7	7/7

[a] Seven positive of seven mice.

4. Discussion

Our study demonstrated that infection of fur mites induced NC mice to develop skin lesions associated with an elevated serum IgE levels. Histological examination revealed that the lesions induced by mite infection appeared to be indistinguishable to the skin lesions in NC kept in conventional conditions as had been reported by Matsuda et al. [1]. However, these mites induced no obvious skin lesions in the other two strains of mice, BALB/c and C57BL/6. Our results are consistent with the previous finding that these mites are usually harmless in most strains of mice [2]. In the NC mouse strain the skin lesions appeared 3–4 weeks after mite infection, and became progressively worse until 8 weeks after infection. Total serum IgE was found to increase up to 16 weeks of age in association with the severity of skin lesions (Fig. 2). These results suggest that mite infection is one of the major agents provoking skin lesions in NC, and the NC strain has a specific background to cause hypersensitivity to fur mite antigen.

Effect of other environmental allergens were neglected in our study because these mice were kept in clean rooms with air filtration. Moreover, the role of mites on IgE hyperproduction was confirmed by the elimination of fur mites by treatment with ivermectin. Ivermectin is a chemically modified form of avermectin, which is similar to the macrolide antibiotics and acts strongly against mites [4,5,7]. Ivermectin spray was chosen as the method of administration because this

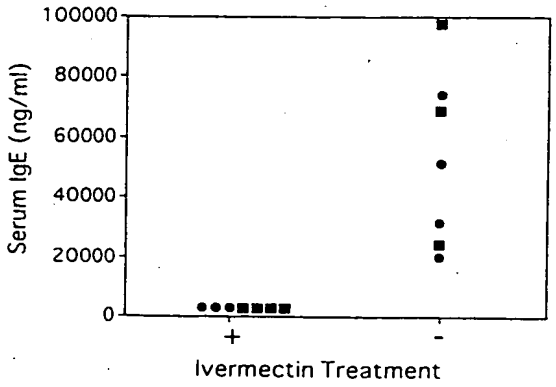

Fig. 4. Serum IgE levels in the mite-infested NC either with or without ivermectin treatment. Four-week-old NC/Kuj which had been born from mite-infected parents were treated with either ivermectin or vehicle, and the serum IgE was determined after 8 weeks. (■) Male NC and (●) female NC.

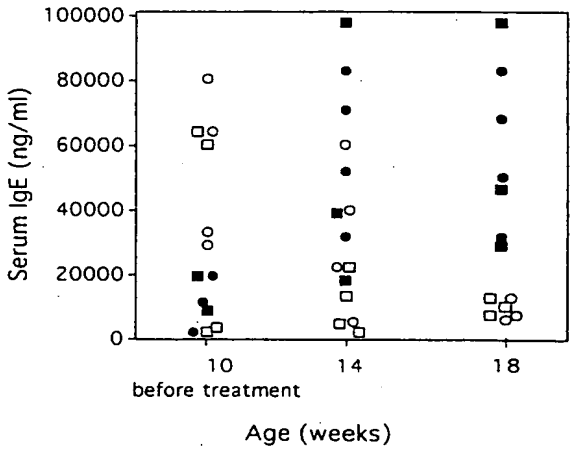

Fig. 5. Ivermectin treatment diminishes the serum IgE levels in mite-infested NC. Ten-week-old mice showing skin lesions which had been infested with fur mites were treated with ivermectin spray and the serum IgE levels were compared with those treated with vehicle spray. (■) Vehicle-treated male NC, (●) vehicle-treated female NC, (□) ivermectin-treated male NC and (○) ivermectin-treated female NC.

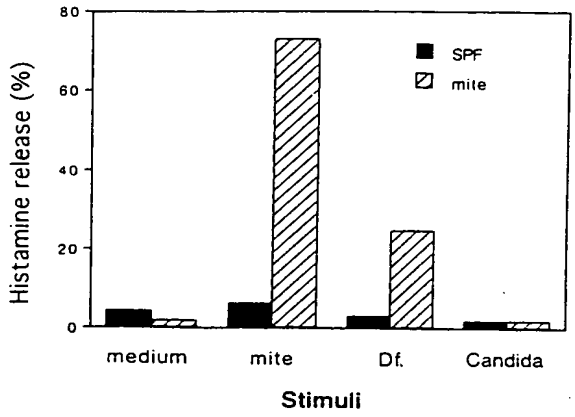

Fig. 6. Fur mite-infested NC has specific IgE to fur mite antigen in the serum. BMMC were sensitized with sera obtained from either SPF-NC or mite-infested NC, and incubated with fur mite extract (protein concentration, 9.4 μg/ml), 10 μg/ml Df antigen or 10 μg/ml *Candida* antigen, and percent histamine release was determined. One typical result was chosen from three independent experiments, which all yielded fundamentally the same results.

method is quick and easy to perform, not stressful for mice and is considered to cause minimal effect on their immune system. Ivermectin treatment of NC before skin manifestation completely arrested the development of dermatitis and the elevation of total serum IgE (Fig. 4), and ivermectin treatment after skin manifestation caused remarkable improvement of skin lesions and significant decrease of serum IgE levels (Fig. 5).

As interleukin-4 (IL-4) is a potent agent inducing IgE production, we examined the serum IL-4 levels in both SPF-NC and mite-infested NC to understand the mechanism of IgE hyperproduction, but found the serum IL-4 levels in NC were below the detection limit (2 pg/ml), suggesting normal serum IL-4 levels in these NC (data not shown). As B cells in NC were shown to produce high amounts of IgE in vitro in response to IL-4 (H. Matsuda, personal communication), an impaired responsiveness of B cells to IL-4 might also play a key role on the pathogenesis of fur mite-induced dermatitis in NC.

Our study also demonstrated the existence c specific IgE antibody to fur mite antigen (*My ocoptes musculinus*) in the sera of mite-infeste NC, indicating that mite-infested NC show imme diate type hypersensitivity to fur mite antiger Interestingly, BMMC sensitized with the sera c the mite-infested NC released less but substantia amounts of histamine in response to Df antiger indicating the cross-reactivity of *Myocoptes mus culinus* antigen with Df antigen.

Atopic dermatitis is a chronic eczematous ski disease, which mostly occurs in infancy and runs course of remissions and exacerbations. An ele vated serum IgE is seen in approximately 80% o patients with atopic dermatitis and the level corre lates well with the severity of the dermatitis. Hy persensitivity to house-dust mites has bee considered to play an important pathogenic rol on the development of dermatitis, because a larg population of patients show strong immediat skin reaction and high titer of serum specific IgE to house-dust mite antigen and studies of patc test reactions induced skin lesions similar to tha of atopic dermatitis [8]. In addition, house-dus mite allergen inhalation was reported to induc exacerbation of atopic dermatitis in challenge tes performed in a double-blind, randomized placebo-controlled fashion [9]. House-dust mit allergens are thus considered to be one of the major agents provoking skin lesions.

Although the role of immediate-type hypersensitivity remains to be determined in causing severe scratch followed by dermatitis in the mite-infected NC, we now provide a new model system o antigen-induced dermatitis, and this is of particular importance for understanding the pathogenesis of atopic dermatitis.

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Inhibition of Airway Inflammation in rDer f 2-Sensitized Mice by Oral Administration of Recombinant Der f 2

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Recombinant Der f 2 (rDer f 2) is a promising new allergen expected to improve the diagnosis and immunotherapy of house dust mite allergy and to further immunological studies. To evaluate the hyposensitizing activity of rDer f 2 to mite allergy, we examined the effect of its oral administration on allergic inflammation in A/J mice immunized with mite allergens. A/J mice immunized with rDer f 2 alone or rDer f 2 + crude mite extract were orally given 0 (control), 0.01, 0.1, or 1 mg/day of rDer f 2 for 4 weeks, followed by an antigen inhalation challenge. Twenty-four hours after rDer f 2 inhalation, control animals experienced severe leukocyte influx into the airway. The infiltrating cells were mainly neutrophils, with some eosinophils and lymphocytes. The concentrations of IL4, IFNγ, and soluble ICAM-1 in the bronchial alveolar lavage fluid increased twofold compared with values before rDer f 2 inhalation. In contrast, inflammation was significantly suppressed in mice given 1 mg/day of rDer f 2 orally for 4 weeks and partially suppressed in those fed 0.1 mg/day of the antigen. Plasma anti-rDer f 2 antibody levels were unchanged by oral rDer f 2 treatment. Mite extract inhalation challenge provoked neutophilia in rDer f 2 + mite-sensitized control mice, and this allergic reaction tended to decrease in sensitized mice fed 1 mg/day of rDer f 2 orally for 4 weeks. We conclude that rDer f 2 has hyposensitizing activities and may be useful in immunotherapy for house dust mite allergy. © 1997 Academic Press

INTRODUCTION

To date, several clinically important allergens has been identified in body and culture extracts of the house dust mite, *Dermatophagoides farinae* (1, 2). Among these, Der f 2 has had its structural gene cloned, and the product of this cloned gene (rDer f 2) was shown to exhibit allergic activity in humans comparable with native Der f 2 prepared from mite extract (3). Therefore, rDer f 2 is potentially useful in allergic immunotherapy.

Evidence supports that the late-phase asthmatic reaction (LAR), but not the immediate reaction, closely resembles the natural history and severity of certain allergic diseases (4–6). LAR occurs 6–48 hr after allergen challenge and is always accompanied by severe infiltration of leukocytes into airway tissue. Such reactions are long lasting and sometimes difficult to manage. While administration of nonbronchodilator or prophylactic drugs is commonly used in the treatment of LAR, immunotherapy with sensitizing allergens has also been tried (5–7).

In the present study, we examined the effect of oral rDer f 2 administration on antigen-induced airway inflammation characterized by leukocyte influx in a mite-sensitized A/J mice strain highly responsive to mite allergens.

MATERIALS AND METHODS

We performed two experiments. In experiment 1, mice were immunized and challenged with rDer f 2 alone. In experiment 2, mice were cosensitized with rDer f 2 and crude mite extract and challenged with crude mite extract. Animals were fed PBS or rDer f 2 solution in both experiments (Fig. 1).

Antigen

Recombinant Der f 2, clone 1 (3), was kindly supplied by the Nikka Whisky Distilling Co., Ltd. (Chiba, Japan). Crude body extract from house dust mite, *D. farinae* (mite extract), was kindly supplied by Torii & Co., Ltd. (Chiba, Japan). This mite extract contained about 0.3% (w/w) of native Der f 2, as determined by enzyme-linked immunosorbent assay. Allergens were dissolved in PBS before use.

Animals

Male A/J mice, 6 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan).

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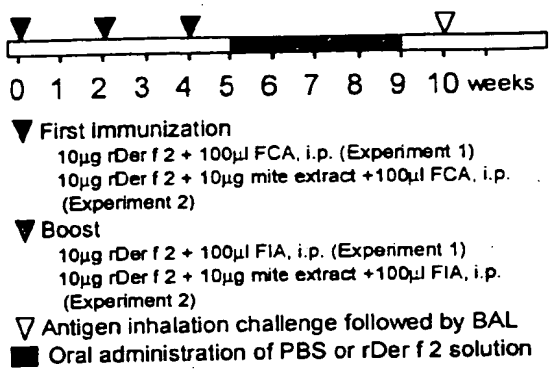

FIG. 1. Experimental schedule. rDer f 2-sensitized A/J mice were challenged with rDer f 2 in experiment 1. Mice coimmunized with rDer f 2 and mite extract were challenged with mite extract in experiment 2. Animals were fed PBS or rDer f 2 in both experiments during the administration period.

Experiment 1

Immunization. Allergen solution containing 10 μg of rDer f 2 and 100 μl of Freund's complete adjuvant (FCA) was injected intraperitoneally into 7-week-old A/J mice as the first immunization. After 14 and 28 days, 10 μg of rDer f 2 and 100 μl of Freund's incomplete adjuvant (FIA) were given intraperitoneally as a booster.

Several mice without immunization ("naive mice") were also used as reaction-negative controls.

Experimental groups and oral administration of rDer f 2. rDer f 2-sensitized mice were divided into three rDer f 2-fed groups (0.01, 0.1, and 1 mg/day rDer f 2) and a PBS-fed group (as control). Each group consisted of 7–10 animals. Oral administration was started 7 days after the last immunization. Animals were given 0.25 ml of rDer f 2 solution or PBS 5 sequential days a week for 4 weeks using a syringe fitted with an 18-gauge stainless sound. Mice in the 3 rDer f 2-fed groups received 0.01, 0.1, or 1 mg/body wt/day of rDer f 2.

Naive mice received no treatment over the experimental period.

Induction of allergic airway inflammation. An antigen inhalation challenge followed by bronchial alveolar lavage was carried out 3 days after the last oral administration. Mice were challenged with rDer f 2. Leukocyte numbers and biochemical activities in the bronchial alveolar lavage fluid (BALF) were determined as an index for allergic airway inflammation.

An aerosol mist of 10 mg/ml rDer f 2 solution was generated by an ultrasonic nebulizer and inhaled by a mouse confined in a small chamber for 30 min. After 6 or 24 hr, the animals were anesthetized by an excess amount of pentobarbital sodium (100 mg/kg, ip) and killed by bleeding. Tracheal cannulation was then performed with a PE-50 polyethylene tube. PBS (0.7 ml) was injected into the lung through the tube, followed by the recovery of BALF. This procedure was repeated once more to yield 1.0–1.2 ml of BALF from each mouse. After counting the total cell number in the BALF, cytospin preparations were made from 0.2-ml aliquots of the BALF. The numbers of macrophages, neutrophils, eosinophils, and lymphocytes were determined by differential leukocyte counting after Difquick stain, in which we examined about 300 cells/preparation. A part of the BALF was centrifuged at 1500 rpm for 15 min, and its supernatant was examined for "biochemical values," that is, total protein content, lactate dehydrogenase (LDH), and β-glucuronidase (GLU) activities and IL4, IL6, TNFα, IFNγ, and soluble ICAM-1(sICAM-1) concentrations. Total protein, LDH, and GLU were determined by using an automatic enzyme analyzer (Fuji Dry-Chem, Fuji Photo Film Co., Ltd., Japan). Cytokines and sICAM-1 were measured by enzyme linked immunosolvent assays (ELISAs). It has been shown that total protein concentration and LDH and GLU activities in the BALF increase when airway is inflamed by physical/chemical damage (8, 9) and infection (10–12). Increased levels of proteins and LDH are mainly derived from damaged epithelia and leaked plasma (13, 14). GLU is released by activated neutrophils and macrophages (11, 15). Increases in cytokine and sICAM-1 concentrations in airway lavage fluids during allergic inflammations have also been reported (16–19). sICAM-1 are shed from the cell surface over expressing ICAM-1 molecules after activation (16, 20).

Determination of cytokine and sICAM-1 concentrations in BALFs. Cytokines and sICAM-1 levels were determined by ELISAs after recommendations from manufacturers of antibodies. We used following combinations of capturing and detecting antibodies: rat monoclonal anti-mouse IL4 (PM-18034A, PharMingen, San Diego, CA) and biotinylated rat monoclonal anti-mouse IL4 (PM-18042D, PharMingen); rat monoclonal anti-mouse IL6 (PM-18071D, PharMingen) and biotinylated rat monoclonal anti-mouse IL6 (PM-18082D, PharMingen); rat monoclonal anti-mouse IFNγ (PM-18181D, PharMingen) and biotinylated rat monoclonal anti-mouse IFNγ (PM-18112D, PharMingen); rat monoclonal anti-mouse TNFα (PM-18131D, PharMingen) and biotinylated rabbit polyclonal anti-mouse TNFα (PM-18352D, PharMingen); rat monoclonal anti-mouse ICAM-1 (270650, Seikagaku Corporation, Tokyo, Japan) and hamster polyclonal anti-mouse ICAM-1 (PM-01541D, PharMingen). Detecting antibodies were labeled by avidin - conjugated β - galactosidase (A - 2300, Vector, Burlingame, CA) or peroxidase-conjugated goat anti-hamster IgG (14-22-06, Kirkegaard & Perry Labora-

tories Inc., MD) for fluorescence or color development. Standard curves for determining sICAM-1 levels were obtained from the BALF at 24 hr after rDer f 2 inhalation of the PBS-fed group. Standard curves for cytokines were made by using recombinant mouse IL4 (PM-19231W, PharMingen), IL6 (PM-19251V, PharMingen), IFNγ (PM-19301U, PharMingen), and TNFα (PM-19321T, PharMingen).

Determination of plasma anti-rDer f 2 IgG levels. Heparinized blood was collected from the retroorbital plexus of individual mice on the day before the beginning of oral feeding and the day after the end of feeding in experiment 1. Plasma (about 20 μl/animal) was separated from the whole blood and frozen until we measured IgG. Anti-rDer f 2 IgG1 and IgG2a were determined by ELISAs using rDer f 2 as a capturing antigen and two detecting antibodies, biotinylated rat monoclonal anti-mouse IgG1 (PM-05002D, PharMingen) and anti-mouse IgG2a (PM-05022D, PharMingen) as described elsewhere (3). Standard curves for determining IgG levels were obtained from the pooled plasma of the PBS-fed group collected before oral administration. The IgG levels of this plasma were taken as "1.0."

Statistical analysis. All the values were expressed as means with standard error. Statistical significance of difference was determined by Student's t test. A P value lower than 0.05 was considered significant.

Experiment 2

Except for the allergen used and several points mentioned below, the same procedures as in experiment 1 were employed.

Immunization. Allergen solution containing 10 μg of mite extract, 10 μg of rDer f 2, and 100 μl of FCA was injected intraperitoneally into 7-week-old A/J mice as the first immunization. After 14 and 28 days, 10 μg of rDer f 2 and 100 μl of FIA were given intraperitoneally as a booster.

Experimental groups and oral administration of rDer f 2. Mice coimmunized with rDer f 2 and mite extract were divided into two rDer f 2-fed groups (0.1 and 1 mg/day rDer f 2) and a PBS-fed group (as control). Animals were fed PBS or rDer f 2 solutions for 4 weeks. Mice in the two rDer f 2-fed groups received 0.1 or 1 mg/body wt/day of rDer f 2.

Induction of allergic airway inflammation. Aerosol mist was generated from 10 mg/ml of crude mite extract and inhaled by mice for 30 min. Leukocyte numbers in the BALF were determined 24 hr after the inhalation challenge. Biochemical activities were not determined in experiment 2.

RESULTS

Side Effects Induced by rDer f 2 Administration

No anaphylactic response was observed in mice given rDer f 2 orally over the experimental period in either experiment 1 or experiment 2.

Time Course of Airway Inflammation after Antigen Inhalation

In a preliminary examination, we determined the time course of the airway inflammation in mice sensitized with rDer f 2 5 min (as 0 hr), 3, 6, 12, 24, and 48 hr after an inhalation of 10 mg/ml of rDer f 2 for 30 min. BALFs were prepared from four to six mice for each time point, leukocytes were counted, and cytokine and sICAM-1 concentrations were measured. Total leukocyte numbers in the BALF began to increase within 3 hr and maximized at 24–28 hr (two- to threefold increase compared to the base value at 0 hr), showing characteristics of late-phase inflammation (Fig. 2). The increasing cells were mainly neutrophils, with some eosinophils and lymphocytes. No such leukocyte influx into the airway was observed in the naive mice challenged with rDer f 2 or the sensitized mice subject to PBS or ovalbumin inhalation. The results suggested that the reaction was antigen specific. We also traced the changes in IL4, IL6, IFNγ, TNFα, and sICAM-1 levels of the BALFs after rDer f 2 challenge in sensitized mice. Each cytokine concentration had a different rise-and-fall pattern. TNFα and IL6 levels, known to be produced by several cell types including mast cells and macrophages, rose and fell rapidly. T-cell-specific cytokine, IL4, and IFNγ levels increased relatively late. sICAM-1 levels began to increase within 3 hr after allergen inhalation and remained high for over 48 hr with a time course that resembled the neutrophil count.

Effect of rDer f 2 Feeding on Allergic Inflammation in rDer f 2-Sensitized Mice in Experiment 1

The BALF of rDer f 2-sensitized mice fed PBS or rDer f 2 solutions was examined 6 or 24 hr after rDer f 2 inhalation in experiment 1. We chose the 6th hour for determining TNFα and IL6, and the 24th hour for examining overall inflammation, IL4, and IFNγ. In the PBS-fed group, total leukocyte numbers in the BALF were increased about twofold at 6 hr and about threefold at 24 hr after the challenge in comparison with naive mice (Fig. 3a). The protein content, the LDH and GLU activities (Fig. 3b), and the IL4, IFNγ, and sICAM-1 (Fig. 3c) levels in the BALF collected from PBS-fed animals 24 hr after inhalation also showed two- to threefold increases compared with naive mice. TNFα and IL6 levels 6 hr postchallenge in PBS-fed mice were 25–50% higher than that of naive mice (Fig. 3c). In

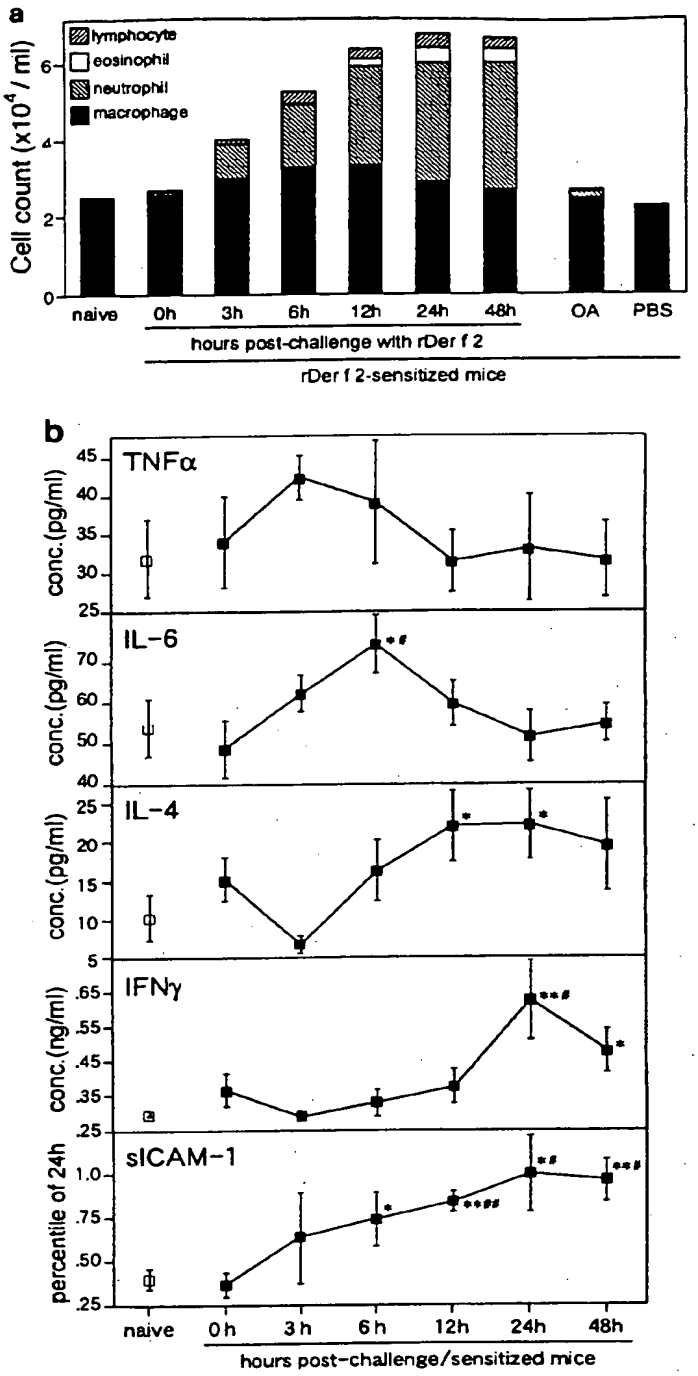

FIG. 2. (a) Time course of the infiltration by leukocytes into the airway after rDer f 2 inhalation for 30 min in rDer f 2-sensitized mice ($n = 4-6$ for each time point). Naive mice ($n = 5$) were examined 24 hr after the rDer f 2 inhalation challenge. Cell counts in sensitized mice challenged with PBS ($n = 4$) and ovalbumin ($n = 4$) are shown as "PBS" and "OA," respectively. Values were determined 24 hr after challenges. (b) Time course of IL4, IL6, TNFα, IFNγ, and soluble ICAM-1 in the BALF. BALF was obtained from the same animals as in (a). $^{**}P < 0.01$, $^{*}P < 0.05$ compared with naive. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ compared with 0 hr of sensitized animals.

contrast, these indexes of inflammation at 24 hr after rDer f 2 inhalation were suppressed in rDer f 2-fed groups in a dose-dependent manner compared to the PBS-fed group (Figs. 3a–3c). A significant difference was obtained in neutrophil count, protein content, LDH activities, IFNγ concentration, and sICAM-1 concentration between the 1 mg/day rDer f 2-fed group and the PBS-fed control group. Biochemical values and neutrophil numbers in sensitized animals correlated closely as shown in Table 1. Neutrophil count and TNFα and IL6 levels 6 hr postchallenge in the 1 mg/day rDer f 2 group were also lower than that of control. The difference was not significant, however.

Changes in Plasma IgG Titer in Experiment 1

Table 2 shows plasma anti-rDer f 2 IgG levels before and after oral administration in experiment 1. Anti-rDer f 2 IgGs were not detected in naive mice. rDer f 2-feeding did not change plasma IgG levels in immunized mice. Neither IgG1 nor IgG2a titer showed close relationships with BALF cell count and biochemical values (Table 1). Anti-rDer f 2 IgE was also detected in sensitized mice by ELISA. However, IgE levels were not high enough to analyze in sensitized animals (Data not shown).

Effect of rDer f 2 Feeding on Allergic Inflammation in Mite-Sensitized Mice in Experiment 2

Mice immunized with rDer f 2 and mite extract in experiment 2 were challenged with mite extract. Inhalation of mite extract provoked late-phase airway inflammation characterized by neutrophil influx in mite-sensitized A/J mice. The severity of the inflammation as judged from the leukocyte count of the BALF was lower than that of the rDer f 2-induced reaction in experiment 1. The BALF neutrophil count in the PBS-fed group were $3.2 \pm 0.5 \times 10^4$ cells/ml, while that in naive mice was $0.2 \pm 0.0 \times 10^4$ cells/ml. The 0.1 and 1 mg/day rDer f 2 groups showed lower neutrophil number than control group (Fig. 4). The difference was not significant, however.

DISCUSSION

Studies in humans and experimental models have shown that oral hyposensitization therapy can suppress cellular immunity including late-phase asthmatic reaction (5, 21–26). In the present study, we examined the oral-hyposensitizing activity of rDer f 2 to mite-allergic inflammation in a murine model. Allergic neutrophil influx in rDer f 2-sensitized mice was suppressed by daily rDer f 2 feeding, suggesting the beneficial effect of oral-hyposensitization therapy with rDer f 2. rDer f 2-feeding also suppressed mite extract-induced reactions in mice immunized with mite extract

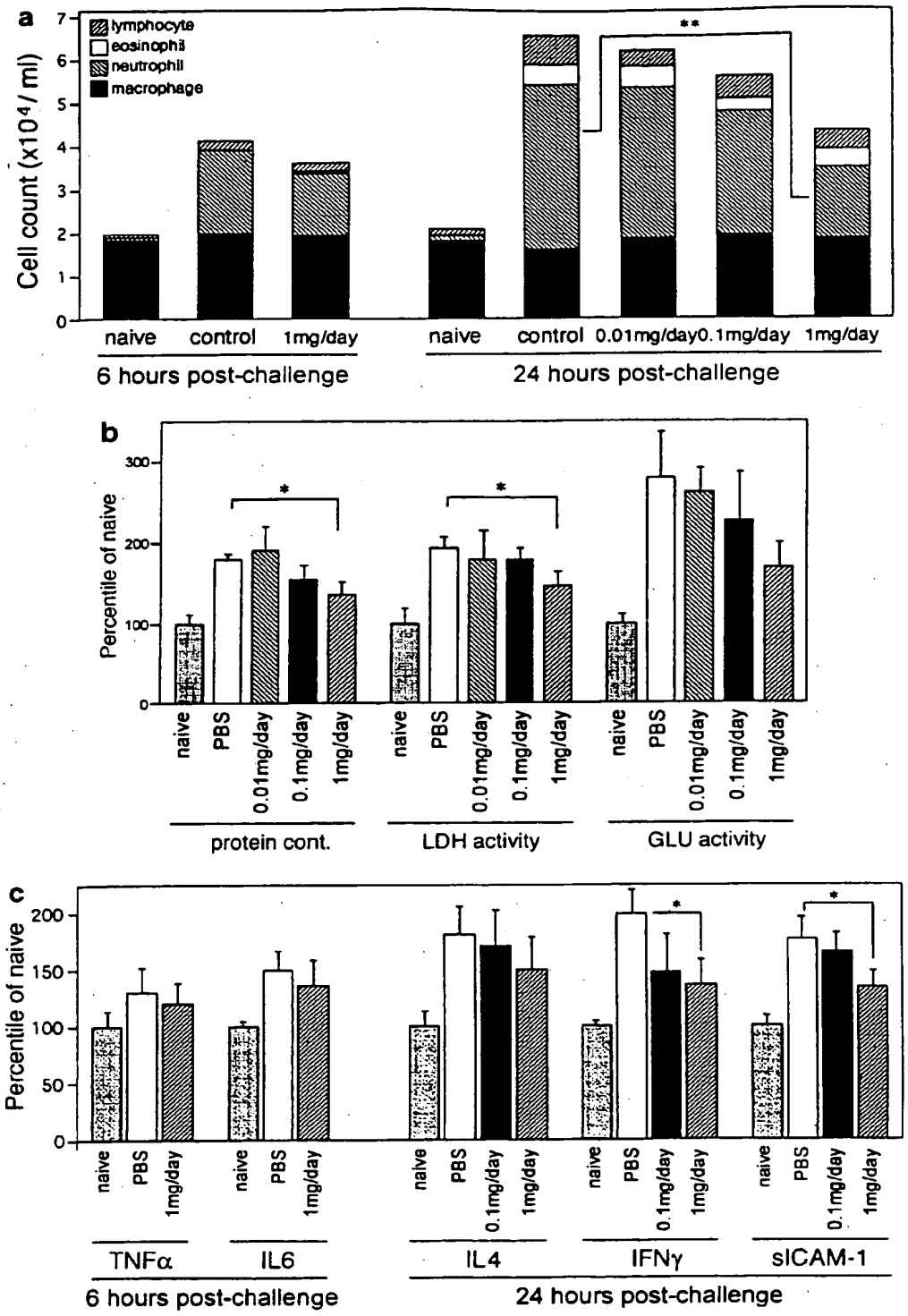

FIG. 3. The effect of oral rDer f 2 administration on (a) leukocyte counts, (b) biochemical activities, and (c) cytokines in the BALF in experiment 1. BALF was collected 6 hr after the rDer f 2 challenge from PBS-fed (n = 7), 1 mg/day (n = 7), and naive mice (n = 6), or 24 hr postchallenge from PBS-fed (n = 10), 1 mg/day (n = 7), 0.1 mg/day (n = 7), 0.01 mg/day rDer f 2-fed (n = 10), and naive mice (n = 8). $^{**}P < 0.01$. $^{*}P < 0.05$.

TABLE 1

Statistical Relationships between BALF Cell Counts, Biochemical Values, and Plasma IgG Levels in Sensitized Mice

	Np	Ep	Protein	LDH	GLU	IgG1	IgG2a	IL4	IFNγ	sICAM-1
Neutrophil		0.43	0.75	0.82	0.55	0.22	−0.04	0.37	0.51	0.57
Eosinophil	ns		0.48	0.43	0.24	−0.09	0.11	−0.10	0.19	0.29
Protein	$P < 0.01$	$P < 0.05$		0.94	0.63	0.21	0.04	0.24	0.48	0.57
LDH	$P < 0.01$	ns	$P < 0.01$		0.71	0.32	−0.04	0.31	0.47	0.62
GLU	$P < 0.05$	ns	$P < 0.01$	$P < 0.01$		0.43	−0.03	0.05	0.18	0.67
IgG1	ns	ns	ns	ns	ns		−0.34	−0.01	−0.28	0.21
IgG2a	ns	ns	ns	ns	ns	ns		−0.26	0.10	−0.30
IL4	ns	ns	ns	ns	ns	ns	ns		0.59	0.18
IFNγ	$P < 0.05$	ns	$P < 0.05$	$P < 0.05$	ns	ns	ns	$P < 0.01$		0.38
sICAM-1	$P < 0.01$	ns	$P < 0.01$	$P < 0.01$	$P < 0.01$	ns	ns	ns	ns	

Note. LDH, lactate dehydrogenase; GLU, β-glucuronidase; IgG1, IgG2a, plasma levels after oral administration. Each corelation coefficient was calculated from the measurements of 20 sensitized mice in PBS and 1 mg/day rDer f 2 groups in experiment 1.

and rDer f 2. Since Der f 2 constituted only 0.3% of the provoking antigen (mite extract), a more intense sensitization with rDer f 2 might provide better results. Selection of mite-allergic patients whose major sensitizing-allergen is Der f 2 will be needed to achieve adequate levels of hyposensitization with rDer f 2.

The airway inflammation observed in the A/J mice in the present study is characterized by a severe infiltration of neutrophils into the airway. The concentration of protein, LDH, and GLU in the BALF, which could be derived from damaged cells or leaked plasma, showed a close relationship with neutrophil number. Although the role of eosinophils seems to be highlighted more than that of neutrophils in recent studies on the development of late-phase inflammation (7, 27), human and animal studies have shown that neutrophils contribute to severe airway injury and hyperresponsiveness by infiltrating inflammatious sites and releasing a variety of substances including oxygen metabolites, proteases, cationic materials, and potent lipid mediators (14, 15). Thus inhibition of the allergic inflammation caused by neutrophils is thought to play a significant role in the efficacy of oral immunotherapy by rDer f 2.

Several studies have demonstrated a role for anergy, clonal deletion, and active suppression in oral tolerance and oral hyposensitization (22, 30, 31). We performed a kinetic analysis of cytokines and sICAM-1 in BALF to elucidate the mechanism of the immunotherapy. Neutrophil influx is very efficient under conditions of the activation of cell adhesion molecules, especially ICAM-1 in the target tissues (32). sICAM-1 levels in the BALF, which could reflect the status of the expression and activation of ICAM-1 in airway tissues (20), increased synchronously with neutrophil counts after rDer f 2 inhalation in sensitized mice over 48 hr. While the influences of IL4 and IL6 on the expression of ICAM-1 are not clear, TNFα and IFNγ have been shown to be strong inducers of ICAM-1 in various tissues in both humans and mice (33–35). Since the peak

TABLE 2

Plasma Anti-rDer f 2 IgG1 and IgG2a Levels before and after Oral Administration

	Before	After
IgG1		
PBS	1.00 ± 0.09	0.93 ± 0.12
0.01 mg/day	0.91 ± 0.07	1.07 ± 0.23
0.1 mg/day	1.17 ± 0.21	1.14 ± 0.24
1 mg/day	1.14 ± 0.14	0.93 ± 0.12
IgG2a		
PBS	1.00 ± 0.17	1.69 ± 0.38
0.01 mg/day	1.11 ± 0.32	1.55 ± 0.53
0.1 mg/day	1.21 ± 0.17	1.70 ± 0.71
1 mg/day	1.03 ± 0.27	1.30 ± 0.31

Note. Both the IgG1 and IgG2a titer of the PBS-fed group before oral treatment were about 1/4000.

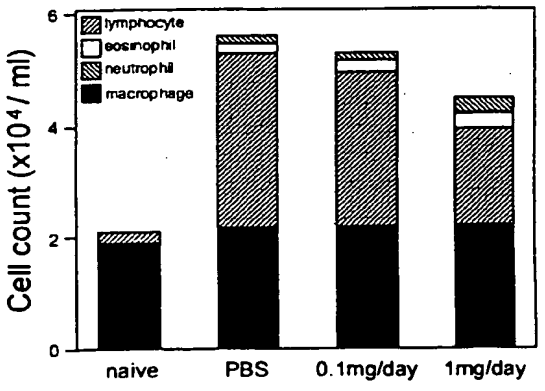

FIG. 4. The effect of oral rDer f 2 administration on leukocyte counts in the BALF collected 24 hr after inhalation of crude mite extract in experiment 2. Seven mice were examined for each group.

TNFα and IFNγ levels in BALFs (at 3 and 24 hr post-challenge, respectively) had a time lag between them, these two cytokines might share in the 48-hr-activation of ICAM-1 in this allergic model. Several studies have reported a beneficial role for IFNγ but unfavorable roles for Th2-type cytokines including IL4 and IL6 in the eosinophil-dominant late-phase reaction and immediate reaction (36, 37). On the other hand, IFNγ can play a crucial role in the pathogenesis of allergic reactions caused by cellular immunity (38, 39). It is not easy to determine which type of cytokine, Th1 or Th2, participated in the development or suppression of the complicated allergic inflammations in the present study. From the BALF, we found that all the levels of cytokines and sICAM-1 measured at 6 or 24 hr post-challenge decreased in the rDer f 2-fed animals compared to PBS-fed mice. Lower expression of cytokines could result in lower expression of adhesion molecules and less severity in leukocyte influx in the airway. It was suggested that anergy or clonal deletion played an important role in the suppression of airway inflammation by rDer f 2 feeding in rDer f 2-sensitized mice. Since both Th1 and Th2 type cytokines can induce an allergic reaction, suppression of both types at the same time is better than active suppression, in which certain kinds of cytokines are activated as "suppressors." rDer f 2 feeding did not affected the plasma anti-rDer f 2 IgG1 or IgG2a levels, or their ratio, reflecting a systemic balance between Th1 and Th2. The suppressive effects against allergic reactions by oral antigen administration might be specific for restricted tissues.

Mite extract contains many antigenic substances and is difficult to biochemically standardize (40, 41). Therefore, ideally, purified mite allergens should be substituted for mite extract in the immunotherapy of house dust mite allergy (42). The recombinant protein of Der f 2, a group 2 major mite allergen, which possesses allergic activity comparable to that of the native allergen, can be produced in large quantities (43). rDer f 2 could prove a useful clinical tool.

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Review article

The mouse ear swelling test (MEST) in the 1990s ☆

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Abstract

The mouse ear swelling test (MEST) was developed in the early 1980s to provide a lower cost, shorter and objectively graded alternative to the existing guinea pig based tests for delayed contact hypersensitivity. In the ensuing time, the test design has been modified and its use has been extended to additional applications (phototoxicity and photosensitisation screening and as a mechanistic tool in studying tumor promoting agents) and to evaluating a broader range of test materials (treated fabrics, medical devices, environmental pollutants, specialty chemicals, drugs, etc.) in the hands of new investigators. Likewise, other murine based tests have been developed. The MEST and one other murine test (the local lymph node assay, or LLNA) have also now been included in the lists of regulatorily accepted sensitization test systems. The current version of the MEST protocol is presented here, along with the rationale for changes, a review of evaluations and interlaboratory trials and an overview of the range of uses to which it has been applied.

Keywords: MEST; Sensitization; Contact sensitization

1. Introduction

Antigen-specific T-cell-mediated (Type IV) hypersensitivity reactions occur from 24 to 48 h after exposure (either epicutaneously, as in tuberculin testing, or dermally, as is the major source of concern occupationally and environmentally). T-lymphocytes which have previously been specifically sensitized to an antigen migrate to the region of exposure 'recruiting' macrophages leading to the accumulation of basophiles and accompanied by mediator release. The result is erythema and edema

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in the region of contact. The erythema is the basis of the traditional guinea pig based sensitization tests. The edema portion of the sensitization response can be detected when the site exposure to an antigen is the skin, but it is not easily distinguished from background and therefore is not generally used as an endpoint marker. When the ear is used as a 'challenge' site, however, a response is very evident and easy to assess quantitatively.

Conventional tests for identifying potential dermal delayed contact sensitizers have a number of disadvantages, such as cost, length of test, a large amount of required animal care space, difficulty is assessing pigmented materials as antigens, and having a subjective (rather than objective) endpoint (Gad et al., 1985a). Several of these disadvantages are reflections of limitations of the guinea pig as a model and the methodology of evaluating response in terms of observing and subjectively 'grading' skin erythema. The mouse ear swelling test (MEST) and variations on it were developed to overcome this disadvantage.

Since Crowle (1959a,b) formally proved that passive transfer of delayed-type contact hypersensitivity can be produced in the mouse in 1959, research immunologists have generated a wealth of information in attempts to understand the delayed-type hypersensitivity (DTH) response in this species (Asherson and Ptak, 1968).

In particular, they have demonstrated that thymus-derived cells are necessary for inducing a DTH response (DeSousa and Parrott, 1969). Also, the mouse has been used to investigate immunosuppressive properties of certain drugs, such as fluorinated steroids and corticosteroids. All of these have led to the development of a formalized test procedure, the MEST.

2. Procedure

The MEST presented here is a modification of the procedure described by Gad et al. (1985, 1986) for evaluating test substances for their potential to cause dermal sensitization in mice. This procedure evaluates contact sensitization by quantitatively and objectively measuring mouse ear thickness. This method is shown diagrammatically in Figs. 1–3.

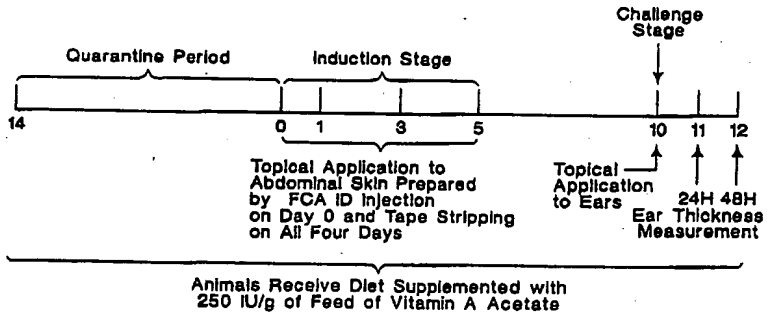

Fig. 1. A line chart of the optimal design for the MEST protocol.

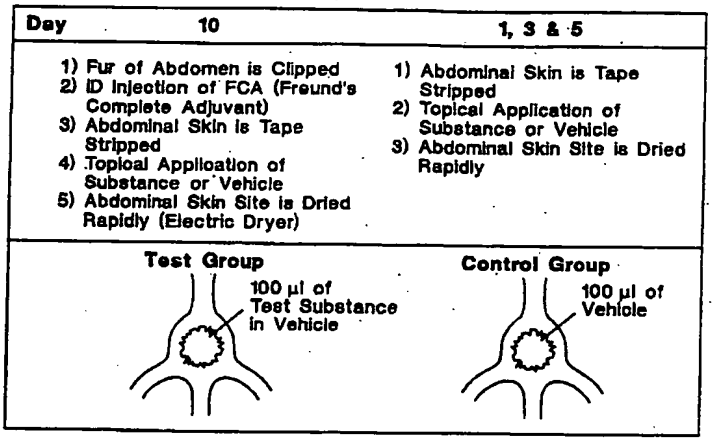

Fig. 2. Details of the induction stage procedures conducted for the MEST test.

2.1. Animals

1. CF-1 or Balb/c female mice, 6–8 weeks old, are used. The mice are observed for at least one week before the start of the study to detect any signs of illness. Mice that show poor growth or signs of illness are excluded from use on a test. Mice are given a diet supplemented with 250 IU/g of vitamin A acetate (Gad, 1993) starting at the time of their arrival.

2. Any mouse displaying redness of either ear prior to the start of a test should be replaced.

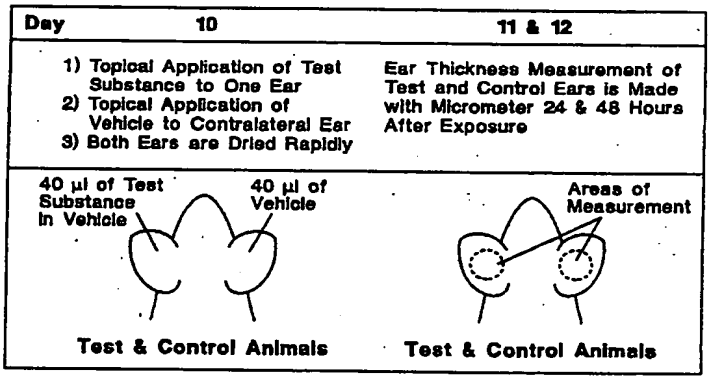

Fig. 3. Details of the challenge stage procedures for the conduct of the MEST test.

3. Mice, which have been randomly placed in cages upon arrival, are assigned to groups (a maximum of five/cage) by labelling cage cards. For each test substance investigated, a pretest group of at least eight mice, a test group of at least 10 mice, and a control group of at least 10 mice are utilized.

4. Because animals are not individually marked, they will always be handled one at a time when each phase of this procedure is performed. The following procedure is conducted to prevent mixing animals during each phase (e.g., shaving, intradermal injections, tape-stripping and dosing). All mice are removed from their original cage and placed in an empty cage for holding. One mouse is removed from the holding cage at a time, the phase activity is performed, and then the mouse is returned to its original cage. This step is repeated for each of the remaining mice in the holding cage.

2.2. Equipment supplies

Required material which must be on hand prior to initiating a study includes the following items. Oditest Model D-1000 thickness gauge (available from the Dyer Co., 1500 McGovernville Rd., Lancaster, PA); latex gloves; Oster small animal clipper with a No. 40 blade (Oster & Co., Cleveland, OH); suitable solvents/vehicles (generally acetone, 70% ethanol, 25% ethanol or 25% methyl ethyl ketone); microliter syringe; glass tuberculin syringe; Dermaclear tape (3R Company, St. Paul, MN); ether; small (50 ml) beaker; anesthesia jar; forms for recording data; 30 gauge needles (1"); Freunds Complete Adjuvant (FCA) (Sigma, St. Louis, MO); small hand vacuum (for collecting shaved hair); vitamin A acetate (Sigma, St. Louis, MO); access to a ventilated fume hood.

2.3. Pretest

1. For 2 weeks prior to initiation of testing, animals are fed a diet enhanced with vitamin A acetate at 250 IU/g of feed.

2. A dermal (abdomen and ear) irritation and toxicity probe study is conducted one week prior to the actual MEST in order to establish the maximum concentration of test substance that produces minimal irritation to the abdomen (belly) region after a single topical application on each of 4 days (if the substance does have potential to irritate skin) and to establish a concentration of test substance that is nonirritating to the ear after a single topical application. Also, dose levels of the test substance that produce systemic toxicity can be identified during the pretest (and subsequently avoided).

3. The test substance is diluted, emulsified or suspended in a suitable vehicle. A vehicle (such as acetone, 70% ethanol, 25% ethanol or methyl ethyl ketone) is selected which should be able to solubilize the test substance and be volatile.

4. Two mice from the pretest group are used to test each concentration of test substance. As many as four concentrations can be evaluated. The same mice used for belly irritation are also used for ear irritation testing. Levels which are irritating to one site (belly or ear) may not be to the other.

5. On day 0, the first day of the pretest, each animal is prepared by clipping the hair from the belly region using a small animal clipper with a size No. 40 blade.

6. After clipping the belly, the outer layers of epidermis (stratum corneum) or each mouse are removed from the shaved belly region with a tacky transparent tape (1-inch wide) such as Dermaclear. This procedure is referred to as 'tape-stripping.' It is not painful and no anesthetic is required. On day 0, the belly skin of each mouse is tape-stripped until the application region appears shiny. While as assistant supports the dorsal portion of the mouse, the tape is pressed firmly over the clipped belly region and quickly removed; this procedure is repeated as many times as needed until the skin appears glossy.

7. After tape-stripping the belly, a volume of 100 μl of solvent containing test substance is applied to the belly region using a microliter pipette. At the same time, test substance is applied to the ventral surface (10 μl) and dorsal surface (10 μl) of the left ear of the mouse using a microliter pipette.

8. On day 1, 24 h after dosing the ears, the thickness of all probe animal ears is measured using an Oditest Model D-1000 thickness gauge.

a. Ether is used to anesthetize the mice in a fume hood while the ears are measured.

b. When a mouse reaches the 'surgical anesthesia' stage, it is removed from the jar and gently placed on the countertop of the fume hood, which is prepared with a protective lining.

c. While supporting the mouse with one hand, the other hand is used to press the finger lever on the Oditest gauge in order to open the flat measurement contacts. One ear of the mouse is then inserted between the contacts until it is positioned with approximately 1–2 mm of the outer edge of the ear showing. After positioning the ear, the finger lever is released to allow the contacts to clamp onto the ear. The measurement is read from the gauge after the indicator needle is stabilized. If desired, one or two more measurements can be rapidly made to be certain of a reproducible reading.

d. Once a reading is obtained, the other (contralateral) ear is measured in the same manner. The animal's body is turned over in order to position the other ear for measurement.

e. All measurements are recorded.

9. On subsequent days, 1, 2 and 3, the belly region is first tape-stripped until shiny and then a volume of 100 μl of test substance is applied topically to the belly region using a microliter pipette.

10. On day 4, 24 h after the last topical application, the belly skin of all animals is observed for dermal irritation. A description of the results is recorded.

11. If any signs of systemic toxicity are observed on any of the pretest days, then they should be noted.

12. Based on the results of the pretest data, a judgement is made as to which concentration will be used for topical induction applications to the belly and for topical challenge application to the ear. A minimal or mildly irritating concentration is preferable for induction so that the potential for achieving sensitization is maximal without harming or compromising the animal. The highest nonirritating concentra-

Table 1
Representative MEST test results

Chemical	MEST results	
	Sensitized	Swelling
Oxazolone	100	134
Toluene dilsocyanate	100	142
DNFB	100	168
N,N-Dimethyl-*p*-nitrosoaniline	100	158
Picryl chloride	90	130
DNCB	80	130
p-Phenylene diamine	67	109
HMDI	67	139
Glutaraldehyde	67	125
Dansyl chloride	60	124
Nickel sulfate	38	118
Methyl methacrylate	44	118
Eugenol	42	119
Hexamethylenimine	40	106
Potassium dichromate	40	114
Methyl ethyl ketoxamine	40	120

tion identified is used for challenge application to maximize responsiveness while avoiding the possibility of having a confounding irritation response.

2.4. Main test: induction stage

Day 0

a. Ten mice are used in the test and ten in the control groups. The belly of each test and control group mouse is clipped free of hair.

b. Immediately after clipping, two i.d. injections of FCA emulsion are made at separate sites in the skin of the shaved belly (each site flanks the ventral mid-line). Approximately 20 μl of FCA emulsion is injected with a glass tuberculin syringe with a 30-gauge needle attached. Injections are performed in test and control mice.

c. Following the i.d. injections, the belly skin of test and control group animals is tape-stripped until the site gives a shiny appearance.

d. After tape-stripping the belly, a volume of 100 μl of test substance (at a concentration determined by pretest) is topically applied to the belly skin of test group animals with a microliter pipette. Control animals receive a dose of 100 μl of vehicle. If greater certainty of identifying weak sensitizers is desired, an additional group of 15 mice is dosed with a concentration one-third of that identified in the pretest. This avoids having false negatives due to being in the down-regulated response region (Thorne et al., 1991).

Days 1, 3 and 5

a. The skin of the belly of test and control group animals is tape-stripped until shiny in appearance.

b. After tape-stripping, a volume of 100 μl of test substance is topically applied to the belly skin of test group animals and a volume of 100 μl of vehicle/solvent is topically applied to control group animals.

2.5. Challenge stage

Day 10

Each test group mouse and each of five control group mice is dosed with 20 μl of a concentration of test substance (determined by the pretest data) on both the ventral and dorsal surfaces of the left ear. The contralateral right ear is dosed with 20 μl of 100% vehicle on the ventral and dorsal sides.

Day 11

Ear thickness measurements are made 24 h after challenge dosing. The procedure described above in sections 8a,b,c and d under pretest is used.

Day 12

Each thickness measurement is made again 48 h after challenge dosing.

2.6. Rechallenge

The inclusion of a rechallenge phase is not mandatory, but rather provides an additional level of sensitivity (as additional induction exposures will with any assay system) and the ability to clarify ambiguous findings if desired. The rechallenge is also useful if the material being evaluated is a mixture; if a positive result is achieved one then wants to evaluate the components of the mixture.

1. If the test substance is judged to be a nonsensitizing agent after the first challenge application, causes dermal sensitization in only a few animals, or causes ear swelling that is weak or questionable, then a second and final challenge application should be performed on each test animal on day 17.
2. The five control group mice from the first challenge are not rechallenged because they have been exposed to the test substance and are no longer true negative controls. The five remaining naive control group animals (not used for the first challenge) are challenged for comparison to the test group animals.
3. The procedure used for the first challenge application will be used for the rechallenged application. Either the same concentration or a new concentration (higher or lower) of test substance may be used, depending on the results of the first challenge.
4. Measurement of both ears is performed on days 18 and 19 (24 and 48 h after rechallenging, respectively). Each thickness measurements is recorded.

2.7. Interpretation of results

1. Judgement concerning the presence or absence of sensitization is made for each animal. The judgement is based on the percent difference (%) between test and control ears. A 'positive' sensitization response is considered to have occurred if the test

ear of one or more animals is at least 20% thicker than the control ear. This effect criterion was selected because it guarantees a level of false positives of less than one in a thousand (Gad et al., 1986).

2. The percentage of animals in a test group that is considered 'positive' is then calculated and recorded as percent responders.

3. The negative control group ear thickness measurements are used to identify any possible dermal irritation reactions, which would be interpreted as false positive dermal sensitization responses.

4. In addition, % ear swelling is calculated for the test group. The left (A) and right (B) ear thickness measurements are added. Percent ear swelling equals that sum of A (test ear thickness) divided by the sum of B (control ear thickness), multiplied by 100.

$$\% \text{ Ear Swelling} = \frac{A}{B}(100)$$

5. When a second challenge application is performed, the data from both challenges are compared. If neither challenge procedure produces a positive sensitization reaction or both produce equivalent responses, the classification of the test substance is based on both challenge applications. If one challenge application (whether it is the first or second) produces a greater number of positive dermal reactions than the other, the classification of the test substance is based on the challenge with the most positive responses.

6. One or more unequivocally positive responses (20% or greater swelling compared to the control ear) in a group of 10 animals should be considered a positive result. A negative, equivocal, or single response indicates that a substance is not a moderate or strong sensitizer.

3. Strengths and weaknesses

The MEST offers distinct advantages compared to the guinea pig dermal sensitization procedures.

1. The mouse is markedly less expensive than guinea pigs.
2. Less vivarium space is required.
3. The duration of the test is shorter.
4. Less test substance is utilized.
5. Due to advantages 1–4, the overall cost of the test is significantly less.
6. The test is objective and quantitative, rather than subjective and qualitative.
7. Materials which stain the skin may be easily evaluated. Several of the materials which have been evaluated were colored and were very difficult to evaluate by existing guinea pig methods.
8. The test has a low false negative rate and no false positive rate, if properly performed (Gad et al., 1986; Stern et al., 1989; Thorne et al., 1991).
9. The test provides a more accurate basis of predicting relative hazard to humans (Gad, 1988).

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10. With the ability to perform rechallenges (not possible in other currently proposed murine tests), one can evaluate component portions of mixtures which have been found to be sensitizers.

3.1. Disadvantages include the following

Fewer people have experience with the test system. Though this test is very robust in its detection of strong sensitizers and in not generating false positives, care in adhering to technique and this study design is important for the identification of moderate and weak sensitizers. The dietary supplementation with vitamin A greatly reduces the variability in test response from one group of animals (or laboratory) to another.

3.2. Test system enhancements

As with all other dermal sensitization procedures, increasing percutaneous absorption will increase test sensitivity. Factors which will increase absorption (and techniques for achieving them) include the following.

1. Increase surface area of solids.
2. Hydrate region of skin exposed to chemical. This can be done by wetting solids and using very occlusive wrapping of application.
3. Irritate induction application site (with 1% sodium lauryl sulfate in water).
4. Abrade application site (this should not be done in combination with irritation of the site).
5. Injection of test material (if possible).
6. Proper selection of solvent or suspending system (see Christensen et al. (1984) for a discussion of the effect of vehicle in the case of even a strong sensitizer).
7. Remove part or all of the 'barrier layer' (stratum corneum) by tape-stripping the application site.
8. Increase the number of induction applications. Though it is not a factor which increases percutaneous absorption, mildly stimulating the immune system of test animals (by such means as injecting FCA (or some other adjuvant) alone or FCA blended with the test material) also increases responsiveness of the test system.

Also, it is generally believed that using the highest possible test material concentration (mildly irritating for induction, just below irritating for challenge) will guarantee the greatest possible sensitization response and will therefore also serve to universally increase sensitivity. There are reports, however (Gad et al. (1985b) for croton oil and Thorne et al. (1986) for isocyanates) that this is not true for all compounds and that a multiple dose (i.e., two or more concentrations) study design would increase sensitivity. Such designs, however, would also significantly increase cost.

As with other biological based assays, concurrent or frequent positive and negative controls are essential to guard against test system failure. A positive control serves to ensure that the system is responsive and will not produce false negatives due to some undetected environmental factors. The negative control does the same

for false positives. Any of these test systems should show 0.05% dinitrochlorobenzene (DNCB) in 70% ethanol to be a strong sensitizer.

3.3. Test limitations and concerns

As with all other biologically based test systems, there are aspects of the MEST which do not meet the needs of situations and individuals or which could be improved. The 'Test System Manipulation' section of this paper attempts to provide a set of tools to address most individual needs for variation in test performance.

Others have tried using radioisotopic incorporation methods allowing measurement of radioactivity levels in the ear (the challenge site) as a 'more objective' or sensitive measure of sensitization (Back and Larsen, 1982), but this both has limitations on use (having to deal with the radioactivity and inability to rechallenge animals in that system) and has not uniformly been shown to improve performance characteristics. The two techniques in combination (ear and isotopic measurement), however, may be more powerful in detecting and characterizing weak sensitizers (Stern et al., 1989).

The original MEST design (Gad et al., 1986) has not been uniformly found to identify weak and moderate sensitizers (Gad et al., 1987; Dunn et al., 1990; Kimber et al., 1990). There is significant evidence that variations in nutrition of test animals (particularly in levels of vitamin A in the diet) can cause significant variability in test response. The method presented here incorporates improvements developed over the last four years which significantly strengthen test performance or at least guard against variability in sensitivity (Johnson et al., 1984; Stadler and Karol, 1984; Thorne et al., 1987; Gad, 1993).

Alternative forms of the MEST (Gad et al., 1987; Descotes, 1988; Kimber and Weisenberger, 1989) that have been proposed used radio-labelled thymidine to tag the lymphocytes (Back and Larsen, 1982; Cornacoff et al., 1988) or called for removing and weighing the ear (Johnson et al., 1984). Neither of these have shown to be more sensitive or reproducible, and both preclude rechallenge.

Another alternative murine-based delayed contact hypersensitivity assay is the local lymph node assay. As the MEST, it has shown to be adaptable to a number of utilizations. Its sensitivity and test performance are seemingly equivalent but like the two variations on the MEST cited above, its design precludes rechallenge. The ability to rechallenge is critical if mixtures are being investigated.

Concurrent or frequent positive and negative controls are essential to guard against test system failure. Any suitable sensitization test system should show 0.05% dinitrochlorobenzene (DNCB) in 70% ethanol to be a strong sensitizer.

3.4. Practical problems and solutions

Virtually all the general problems associated with the use of the current predictive test systems for delayed contact sensitization can be thought of as being an aspect of 'What do the results mean in terms of hazard to people?' These problems arise for a number of reasons, but the major two can be traced back to two facts: first,

as a population, humans will exhibit greater variability in sensitivity than our animal test system; in trying to reduce this gap, our current test systems do not give us a true prediction of relative hazard (i.e., What portion of the human population will be sensitized, and how easily?) in people.

Secondly, what is evaluated in these models may be a mixture, such as a cosmetic. At the same time, if a chemical is found to be a sensitizer, we may be concerned about structurally related compounds evoking a response in those already sensitized to the compound we have tested: that is, that there may be cross sensitization.

Interpretation: once we have animal sensitization test data, we must relate these to potential hazards in humans. On one end of the scale, a negative finding does not guarantee that a material will not be a sensitizer in humans, though most investigators would agree that it is unlikely that such a material would be other than a weak or mild sensitizer.

On the other end, however, it is not nearly as clear what a finding of a material being a strong or extreme sensitizer means in each of these assays. One is presented with two options: In the first, human patch style tests can be performed, and with large enough (100–200) test groups of a representative nature (that is, a variety of ages, skin types and such that resembles that of the population that will be exposed), the results will give one an understanding of what to expect in humans. This approach, however, is expensive and has both ethical and liability concerns of its own.

The second approach is to use a methodology which allows us to evaluate potency in a human model. As Gad et al. (1985a, 1986, 1987, 1988) and Thorne et al. (1987) have pointed out, such potency evaluations require dose response testing and a number of considerations should be taken into account.

Potency: starting with several assumptions, data from four different animal test systems have been used to evaluate one possible procedure for ranking the potency of known sensitizers. These assumptions were:

1. As absolute (100%) responses do not give actual data points (rather, they define a portion of an unlimited response region), only partial (1–99%) response data from animal tests can be used to predict potency.

2. As the probit transformation has already been shown to linearize sensitization dose response data, probit values can be used to adjust different partial dose response values to a comparable basis. This transformation is most stable in the central region (16–84%) of the response range, so partial responses in this range are most desirable.

3. As individual molecules of material evoke the response being both measured and predicted, dose (or exposures) should be expressed on a molecular basis. Accordingly, data should be adjusted for molecular weight.

A method for calculating a potency index should have at least six characteristics.

A. It should be relatively easy to perform, requiring little more than the data and a calculator.

B. It should incorporate data as to test concentration used, incidence of response achieved, and molecular weight of the test material.

C. The resulting potency index should cover a compact scale (say from 0 to 10)

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and not include negative numbers nor cover more than one order of magnitude.

D. There should be a positive correlation between potency and the index number (i.e., more potent compounds should have higher index numbers).

E. The results should serve to separate materials into clear clumps or clusters lending themselves to classification of materials into categories.

F. Data from various test systems should produce similar classification results for compounds and should predict human results which are not contrary to fact.

An earlier attempt at such an index calculating method (Kimber et al., 1991) produced results which were promising, but clearly did not fit desired characteristics C and D. This has been modified as follows (Gad, 1988).

$$\text{Potency index (PI)} = \log \left[\left(\frac{\text{Probit of response index}}{(\text{Test concentration}^*)\,(\text{Molecular weight})} \right) \times 1000 \right]$$

where test concentration is given as a decimal fraction.

This produces results which, as shown below, generally fulfilled the desired design characteristics. A classification scheme based on the resulting index was devised, with the scale as follows:

Class I -PI $>$ 4.0 'Severe'
Class II $-4.0 >$ PI $\geq$ 3.0 'Strong'
Class III $-3.0 >$ PI $\geq$ 2.0 'Moderate'
Class IV $-2.0 >$ PI $\geq$ 1.0 'Mild'
Class V $-1.0 >$ PI $\geq$ 0 'Weak or Questionable'

These can be considered and treated as hazard classifications.

Cross sensitization: a frequent situation is that one member of a structural series will evoke a positive response in those that have been sensitized; we call this broader response 'cross sensitization.' This occurs because the structures of these materials complexed with a protein are not distinguished as different by the 'educated' surveillance lymphocytes.

Any of the animal tests described here can be modified to see if cross-sensitization occurs among members of a series. The test is conducted with multiple groups of animals. Those animals which are successfully sensitized are then rechallenged with other members of the class.

Mixtures: mixtures become a particular problem in sensitization testing because, frequently, we are called upon first to evaluate a complex mixture in an animal test system; then, if it is found to be a sensitizer we are called upon to determine which component is the cause of the positive response. If such a component can be identified, it is frequently possible to reformulate the mixture to serve the desired need without the problem component.

Such components can be identified by continued testing in a set of animals

previously sensitized to the mixture as a whole. Groups of positively sensitized animals are rechallenged with separate samples of different suspect components to identify that which evokes a positive response. The guinea pig methods offer some advantage here, in that multiple components may be simultaneously evaluated on different sites of the same animal.

3.5. Other applications

The MEST provides a basic test paradigm which has a wide range of applications. Reviewing these in depth is beyond the scope of this paper, but a short list of some of the uses that it has been put to or modified for includes the following:

- Basic design can also be used with rats and guinea pigs, or ear challenge and swelling measurement can be used in guinea pig assays for pigmented materials.
- As a model for mechanisms of chemically induced dermal irritation (Patrick et al., 1987).
- Photosensitization model (Kloss et al., 1992)
- Screen for suppression of T-cell-modulated immune response.
- Tool for studying tumor promotion mechanisms (Czerniecki et al., 1988).

Originally, the MEST was employed to evaluate sensitization potential for industrial chemicals only (Gad et al., 1986). It has since found much wider utility, particularly in those areas where a cost effective screen for strong irritants and sensitizers is required (such as for finished fabrics and medical devices).

With the OECD and ISO incorporating the MEST into their lists of regulatorily accepted tests, it should be expected that the test system will see wider utilization.

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Effect of Tacrolimus Hydrate (FK506) Ointment on Spontaneous Dermatitis in NC/Nga Mice

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ABSTRACT—The effect of tacrolimus hydrate (FK506) ointment on spontaneous dermatitis in NC/Nga (NC) mice was examined. FK506 ointment (0.1–1%) suppressed the development of dermatitis and was also therapeutically effective against established dermatitis. Increases in CD4-positive T cells (helper T cells), mast cells, eosinophils and immunostaining of interleukin (IL)-4, IL-5 and IgE were confirmed in the skin of the NC mice, and FK506 ointment suppressed all of these changes. Increased plasma IgE was also confirmed in the NC mice, and treatment with FK506 ointment reduced the plasma IgE level. These results suggested that FK506 suppressed the dermatitis by inhibiting the activation of inflammatory cells and by blocking the cytokine network in the skin of the NC mice. The commercially available steroid ointments showed only marginal effect on the development of dermatitis and showed some signs of side effects such as alopecia or atrophy of the skin. The effect of the steroids might have been masked by these side effects because the steroids showed similar inhibitory effects on the skin histopathological changes and the increase of plasma IgE. From these results, FK506 ointment can be expected to be a useful drug for atopic dermatitis.

Keywords: FK506, Atopic dermatitis, T cell, Mast cell, Interleukin-4

Spontaneous severe dermatitis has already been reported in NC/Nga (NC) mice (1, 2). The mechanisms of the development of this dermatitis has not been clarified, but immunological factors might contribute to its progress (2, 3). We also have observed that dermatitis appeared about 8 weeks after birth when mice were raised in conventional rearing conditions and that plasma IgE increased to high levels along with the development of dermatitis. Histopathological studies also showed that inflammatory cells such as CD4-positive T cells, mast cells and eosinophils were increased in the skin of NC mice (4). Furthermore, it is suggested that both dermatitis and the level of plasma IgE of NC mice are regulated genetically (5). All of these features are very similar to those of patients with atopic dermatitis (6, 7). It is suggested that there is an immunological disorder in NC mice and that such mice may be suitable models of atopic dermatitis in humans.

FK506, a new macrolide immunosuppressive agent (8, 9), is currently used as an immunosuppressant for liver and kidney transplantation worldwide. FK506 inhibits cytokine production of T cells (10, 11) and histamine release from mast cells (12, 13), as well as delayed type allergic reaction in animal models (14) and humans (15). Since these results suggested that FK506 had an inhibitory activity on atopic dermatitis, FK506 ointment has been developed, and found to be effective on human atopic dermatitis (16). In this paper, we describe the effects of FK506 ointment on dermatitis in NC mice and discuss the mechanisms of the action of FK506 and the mechanisms of the appearance of dermatitis in these mice.

MATERIALS AND METHODS

Animals

Male and female NC mice were bred in the Department of Laboratory Animal Science, College of Agriculture, University of Osaka Prefecture.

Drugs

FK506 ointment was prepared at Fujisawa Pharmaceutical Co., Ltd. Corticosteroid ointments betamethasone valerate ointment (Rinderon®-V ointment, 0.12%) and alclometasone dipropionate ointment (Almeta® ointment, 0.1%) were purchased from Shionogi Pharmaceutical Co., Ltd. (Osaka).

Evaluation of inhibitory effect on dermatitis

Effect on developing dermatitis: Five- to eight-week-old NC mice with no skin symptoms were used. One hundred milligrams of ointment was applied to the skin of the head and neck two times a week, on Monday or Tuesday and Thursday or Friday. The severity of dermatitis was assessed once a week by the following scoring procedure: No symptoms, 0; mild inflammation or wound, 1; moderate inflammation or wound or mild hemorrhage, 2; severe inflammation or hemorrhage or ulcer or loss of ears, 3. After about 9 weeks of treatment, the animals were anesthetized with ether and blood was taken by cardiac puncture with heparinized syringes. The number of red blood cells was assessed using a hematocytometer (Sysmex E-4000; Toa Medical Electronics Co., Ltd., Kobe). Plasma IgE, IgG_1 and IgG_{2a} levels were assessed by enzyme immuno-assay according to the previously described method (17) using monoclonal antibody (Yamasa Shoyu Co., Ltd., Choshi).

Effect on established dermatitis: Eleven- to fifteen-week-old NC mice with dermatitis were used. Ointment, scoring criteria and treatment schedule were the same as above.

Histopathological study

Whole heads of the animals were fixed in 10%-buffered formalin solution and decalcified in 10%-formic acid-formalin solution. A block of the forehead skin was removed and embedded in paraffin by the conventional method, cut in 3- and 6-μm sections, and stained with hematoxylin-eosin (HE) and toluidine blue, respectively.

A piece of fresh skin from between the ears of each animal was embedded in OCT compound (Miles, Inc., Elkhard, IN, USA), snap frozen in dry ice-acetone, and then stored at −80℃ until use. Frozen sections, cut in 5-μm slices, were fixed in acetone for 10 min. After pretreatment with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase, the preparations were washed with phosphate-buffered saline (PBS) twice and then treated with blocking medium (10% normal goat serum in PBS). Rat monoclonal antibodies against mouse CD4, interleukin (IL)-4, IL-5 and IgE, purchased from Pharmingen (San Diego, CA, USA), were applied for 1 or 3 hr in PBS with 1% bovine serum albumin; and after washing, goat anti-rat IgG conjugated with peroxidase (Jackson, West Grove, PA, USA) was overlaid for 30 min. Visualization of the reaction products was performed with 3-amino-9-ethylcarbazole. For staining of eosinophils, frozen sections were treated by *O*-phenylene diamine (OPD) after fixation using 10%-buffered formalin. Mast cells stained by toluidine blue were counted by cell number and CD4-positive cells, eosinophils, IL-4, IL-5 and IgE were graded by the following criteria: no staining, 0; slight staining, 1; moderate staining, 2; marked staining, 3; very strong staining, 4.

Statistical analyses

Data was expressed as the mean±S.E.M. Statistical significances of differences were assessed by Dunnett's multiple comparison test following Kruskal-Wallis test or one way analysis of variance and Student's or Aspin-Welch's *t*-test for two sample comparison. P values less than 0.05 were considered statistically significant.

RESULTS

Effects of FK506 ointment and steroid ointments on the development of dermatitis

NC mice, 6- to 8- (FK506 study) or 5- to 8- (steroids study) week-old, with no superficial dermatitis were used. In the untreated mice, dermatitis appeared and increased gradually from the commencement of the study and reached peak levels of inflammation score in weeks 4 to 6 (Figs. 1 and 2). In the ointment base-treated control group, slight inhibition of dermatitis was observed, and

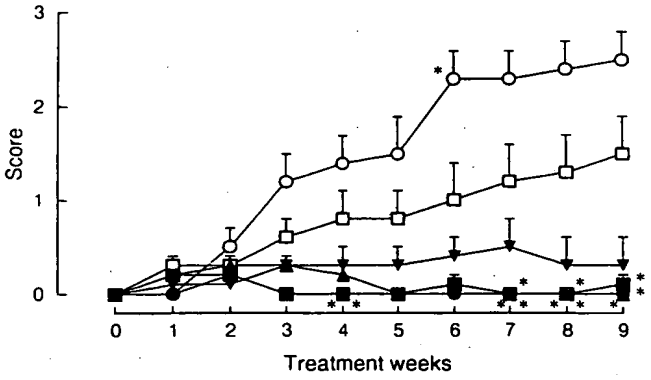


Fig. 1. Effect of FK506 ointment on the development of dermatitis in NC mice. Ointment (100 mg) was applied to the head and neck of 6- to 8-week-old mice for about 9 weeks. ○: no treatment (n=11), □: ointment base (n=12), ■: 0.1% FK506 ointment (n=11), ▲: 0.3% FK506 ointment (n=11), ▼: 0.5% FK506 ointment (n=11), ●: 1% FK506 ointment (n=11). Values are means±S.E.M. *: Significantly different from the ointment base-treated group at $P<0.05$ (Dunnett's multiple comparison test following Kruskal-Wallis test).

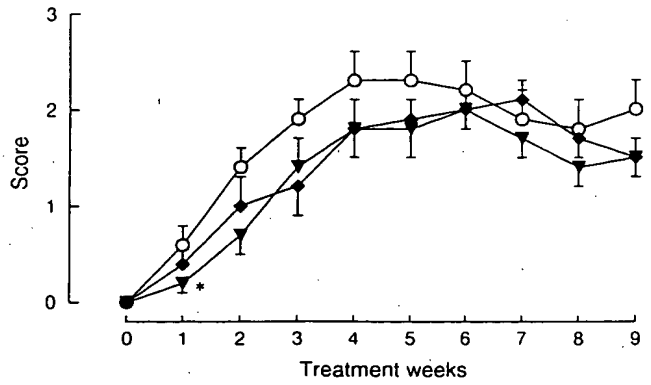


Fig. 2. Effects of steroid ointments on the development of dermatitis in NC mice. Ointment (100 mg) was applied to the head and neck of 5- to 8-week-old mice for about 9 weeks. ○: no treatment (n=16), ◆: 0.12% betamethasone valerate ointment (n=16 until 5th week and n=15 from 6th week onward), ▼: 0.1% alclometasone dipropionate ointment (n=17). Values are means±S.E.M. *: Significantly different from untreated group at $P<0.05$ (Dunnett's multiple comparison test following Kruskal-Wallis test).

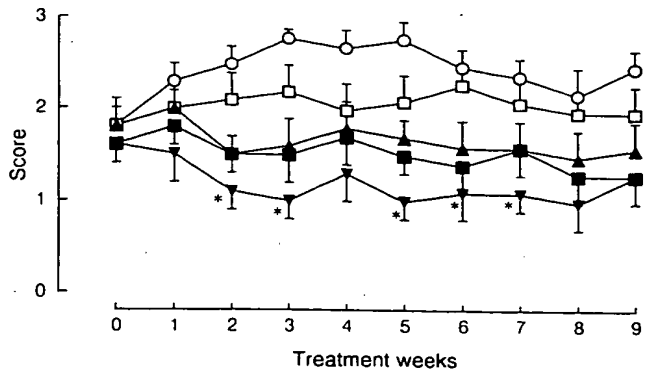


Fig. 3. Effect of FK506 ointment on established dermatitis in NC mice. Ointment (100 mg) was applied to the head and neck of 11- to 15-week-old mice for about 9 weeks. ○: no treatment (n=13), □: ointment base (n=12), ■: 0.1% FK506 ointment (n=13 until 3rd week and n=12 from 4th week onward), ▲: 0.3% FK506 ointment (n=14 until 2nd week, n=13 until 3rd week and n=12 from 4th week onward), ▼: 0.5% FK506 ointment (n=14). Values are means±S.E.M. *: Significantly different from the ointment base-treated group at $P<0.05$ (Dunnett's multiple comparison test following Kruskal-Wallis test).

in all the FK506 ointment (0.1−1%)-treated groups, only slight skin symptoms were observed throughout the observation period (Fig. 1). The two commercially available steroid ointments, betamethasone valerate and alclometasone dipropionate, did not show any clear inhibitory effects (Fig. 2) and even showed some incidence of side effects such as alopecia and atrophy of the skin.

Effect of FK506 ointment on established dermatitis

Mild to severe inflammation of the skin developed in the 11- to 15-week-old NC mice, and the mean score of the symptom in each group was more than 1.5 before commencement of the study. In the FK506 ointment-treated mice, the score decreased gradually during 3 weeks, and significant inhibition was observed in the 0.5% FK506 ointment-treated group compared with the ointment base-treated group (Fig. 3).

Effects of FK506 and steroid ointments on plasma immunoglobulin level and red blood cells

Plasma IgE, IgG_1 and IgG_{2a} levels were increased in the 15- to 17-week-old untreated group compared with younger 6- to 8-week-old animals (Table 1). FK506 ointment showed a concentration-dependent decrease of plasma IgE and also plasma IgG_1, but without concentration

Table 1. Effects of FK506 ointment on plasma IgE, IgG_1 and IgG_{2a} levels in NC mice

Treatment			N	IgE (μg/ml)	IgG_1 (μg/ml)	IgG_{2a} (μg/ml)
Ointment base		(15−17)[a]	12	70.48±14.36	1698±300	2267±427
FK506 ointment	0.1%	(15−17)	11	35.85±14.52	776±156*	1700±121
	0.3%	(15−17)	11	19.46± 7.91**	721±177**	1355±107
	0.5%	(15−17)	11	8.19± 2.91**	837±137*	1660± 72
	1%	(15−17)	11	7.72± 1.68**	981±222	1339±206
No treatment		(15−17)	11	104.39±14.88	1698±231	1921±421
		(6−8)	10	7.14± 3.05##	309± 70##	425± 56##

[a]Figures in parentheses are the age in weeks of mice when blood was taken. Ointment (100 mg) was applied to the head and neck of 6- to 8-week-old NC mice for about 9 weeks. Values are means±S.E.M. *, **: Significantly different from the ointment base-treated group at $P<0.05$ and $P<0.01$, respectively (Dunnett's multiple comparison test following one way analysis of variance). ##: Significantly different from 15- to 17-week-old mice at $P<0.01$ (Student's *t*-test or Aspin Welch's *t*-test).

Table 2. Effects of betamethasone valerate ointment and alclometasone dipropionate ointment on plasma IgE, IgG_1 and IgG_{2a} levels in NC mice

Treatment	N	IgE (μg/ml)	IgG_1 (μg/ml)	IgG_{2a} (μg/ml)
No treatment (14–17)[a)]	16	81.65±7.96	2955±286	2367±214
Betamethasone valerate ointment 0.12% (14–17)	15	32.03±3.39**	2386±247	2307±318
Alclometasone dipropionate ointment 0.1% (14–17)	17	49.30±5.12**	2136±235*	2277±155

[a)]Figures in parentheses are the age in weeks of mice when blood was taken. Ointment (100 mg) was applied to the head and neck of 5- to 8-week-old NC mice for about 9 weeks. Values are means±S.E.M. *, **: Significantly different from the ointment base-treated group at $P<0.05$ and $P<0.01$, respectively (Dunnett's multiple comparison test following one way analysis of variance).

dependency in the latter (Table 1). Both of the steroid ointments also had a decreasing effect on plasma IgE, and alclometasone dipropionate ointment showed a slight inhibitory effect on IgG_1, but betamethasone valerate ointment did not (Table 2). On the other hand, plasma IgG_{2a} was not decreased by FK506 and either steroid ointment (Tables 1 and 2). In the 15- to 17-week-old untreated NC mice a decrease of red blood cells was observed, but FK506 did not show any effect on the cell number (data not shown).

Histopathological study

Even in the younger animals (6- to 8-week-old) that superficially appeared to have no dermatitis, slight infiltration of the cells into the dermis was observed in the section stained by HE. In the untreated animals, 15- to 17-week-old, skin ulcer, thickening of the epidermis and infiltration of many kinds of cells into the dermis was observed (data not shown). In the sections stained by toluidine blue or OPD and immunostaining, mast cells, eosinophils, CD4-positive T cells, IgE, IL-4 and IL-5

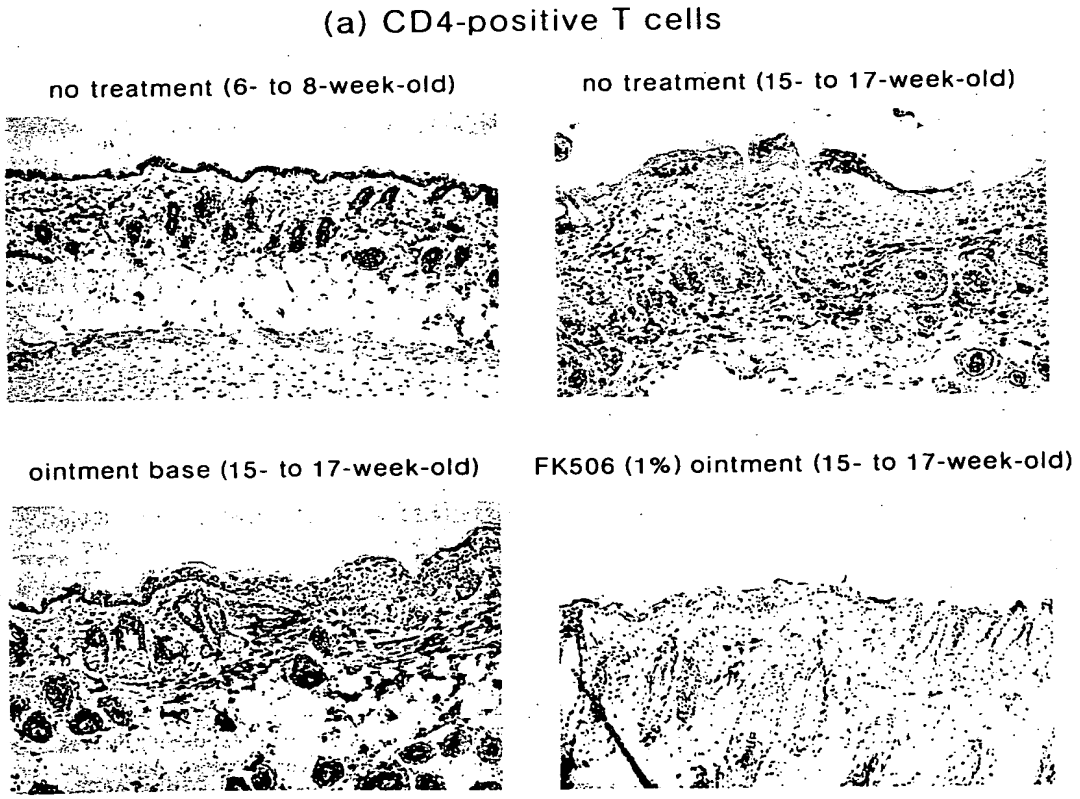


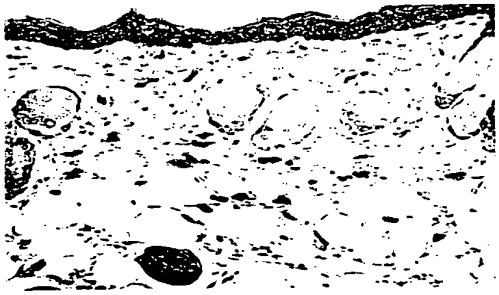
Fig. 4. Effects of 1% FK506 ointment on CD4-positive cells (a), mast cells (b) and IL-4 (c) in dermis of NC mice. Mast cells were stained by toluidine blue; CD4-positive cells and IL-4 were stained by immunostaining.

(b) Mast cells

no treatment (6- to 8-week-old)

no treatment (15- to 17-week-old)

ointment base (15- to 17-week-old)

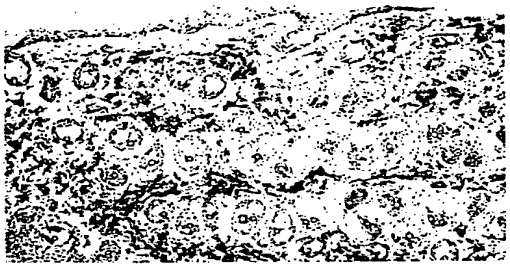


FK506 (1%) ointment (15- to 17-week-old)

(c) IL-4

no treatment (6- to 8-week-old)

no treatment (15- to 17-week-old)



ointment base (15- to 17-week-old)

FK506 (1%) ointment (15- to 17-week-old)

Fig. 4.

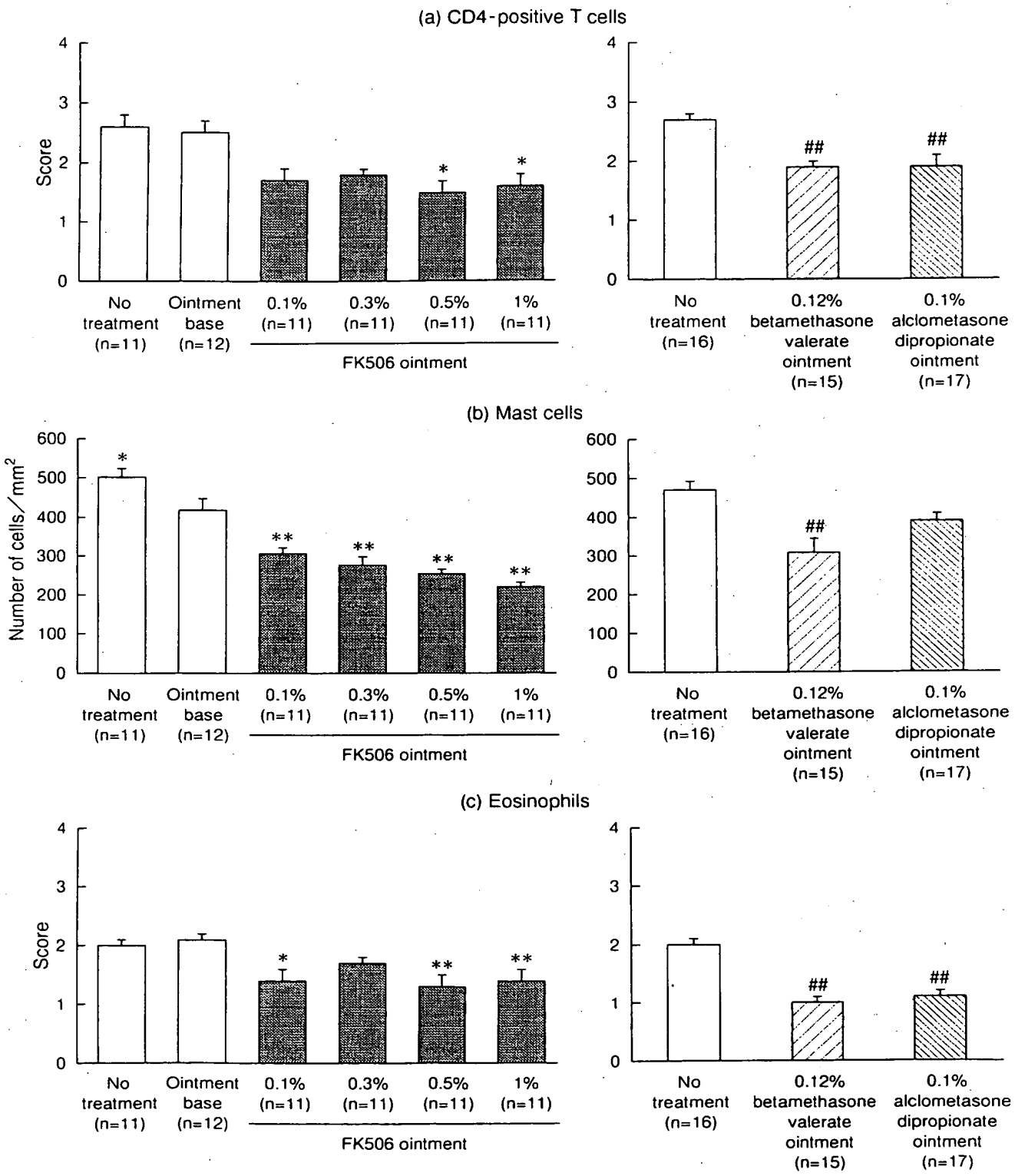


Fig. 5. Effects of FK506 and steroid ointments on CD4-positive T cells (a), mast cells (b) and eosinophils (c) in dermis of NC mice. Values are means±S.E.M. *, **: Significantly different from the ointment base-treated group at $P<0.05$ and $P<0.01$, respectively. ##: Significantly different from the untreated group at $P<0.01$ (Dunnett's multiple comparison test following Kruskal-Wallis test or one way analysis of variance).

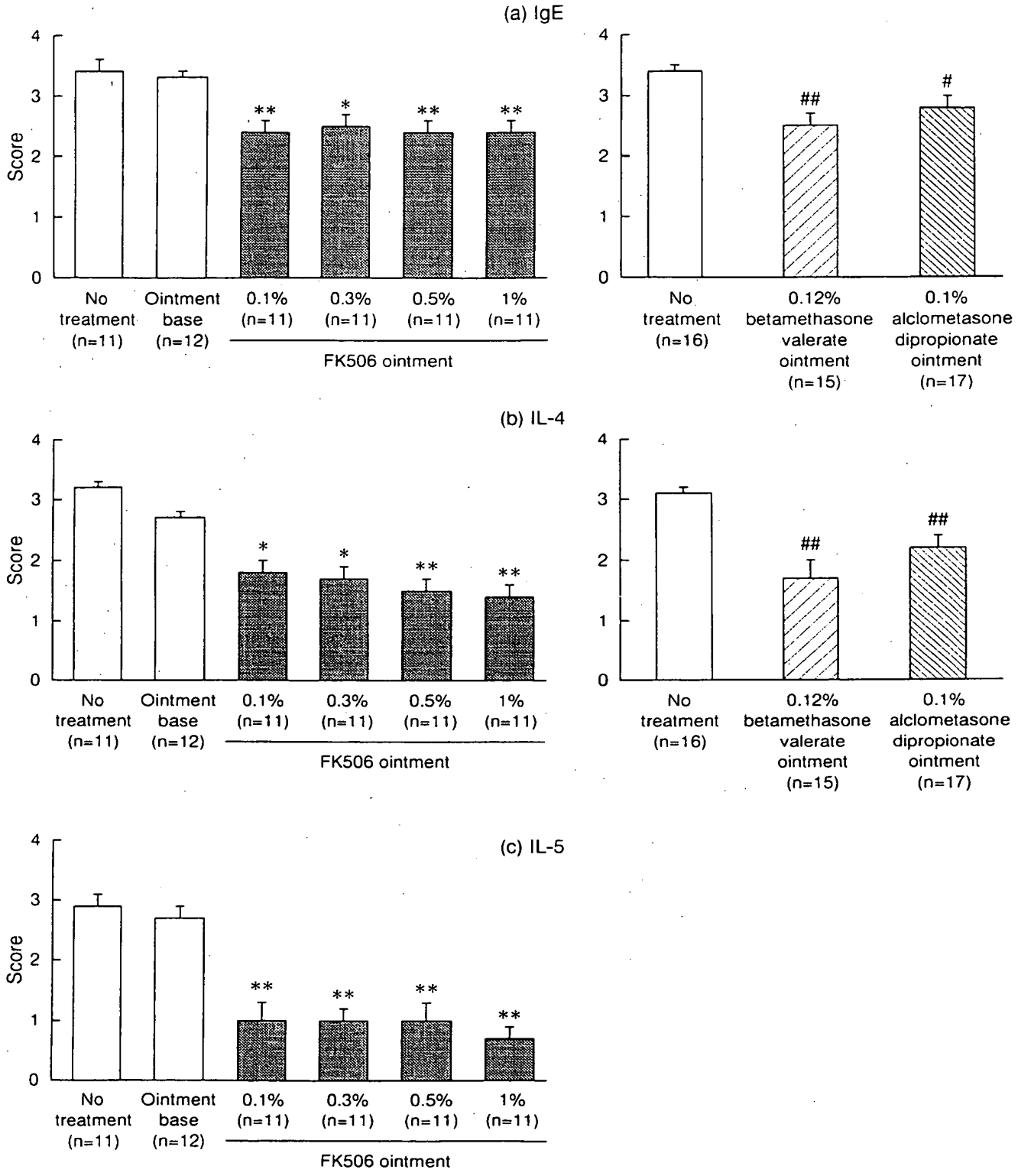


Fig. 6. Effects of FK506 and steroid ointments on IgE (a), IL-4 (b) and/or IL-5 (c) staining in dermis of NC mice. Values are means±S.E.M. *, **: Significantly different from ointment base-treated group at $P<0.05$ and $P<0.01$, respectively. #, ##: Significantly different from untreated group at $P<0.05$ and $P<0.01$, respectively (Dunnett's multiple comparison test following Kruskal-Wallis test).

were increased in the dermis. Typical results of staining of CD4-positive T cells, mast cells and IL-4 are shown in Fig. 4. Treatment with FK506 ointment for about 9 weeks showed an inhibitory effect on all of these changes, but dose-responsiveness was not always observed (Figs. 5 and 6). The steroid ointments showed similar inhibitory effects on most of these features, except IL-5 for which there was no appropriate antibody (Figs. 5 and 6). Since the intensity of immunostaining of CD8-positive cells, IL-2 and interferon (IFN)-γ was very weak even in the dermis of the untreated animals, the effects of the ointments were not studied (data not shown).

DISCUSSION

As we have already reported (4), severe dermatitis developed in NC mice about 8 weeks after birth when the animals were raised in conventional rearing conditions. FK506 ointment showed inhibitory activity on the development of dermatitis and also showed therapeutic effect on established dermatitis in these mice.

As we also reported, pathophysiological assessment of the skin of NC mice showed that the numbers of CD4-positive T cells, mast cells and eosinophils were increased in the dermis of the NC mice (4). We confirmed these changes in this study, as well as an increase in the intensity of immunological staining of IL-4, IL-5 and IgE; and these changes were quantitatively assessed by scoring the grade of the staining. Since these pathophysiological changes are less prominent in younger animals which have no inflammation in the skin, dermatitis and histopathological changes are thought to be correlated with each other.

Atopic dermatitis is reported to be induced by the activation of inflammatory cells such as T cells, mast cells and eosinophils, and the cytokine network of these cells is suggested to be very important (6, 7). From the results of this study, pathophysiological changes of the dermis in NC mice are thought to be very similar to those in human atopic dermatitis patients. FK506 ointment showed the suppressive effects on all of these changes in NC mice, but it was difficult to determine the main target cells of FK506 action from these results. FK506 has been reported to have inhibitory effects on the activation of inflammatory cells, such as T cells (10, 11) and mast cells (12, 13) in animals and humans in vitro. Thus we consider that the clinical effect of FK506 ointment on atopic dermatitis is achieved by inhibition of the activation of these cells.

As reported by Tamada (18), red blood cells were decreased in NC mice. Since FK506 showed no effect on such anaemia in our study, it seemed that FK506 was not effective on some of the pathological changes in these mice.

The two corticosteroid ointments showed only a marginal effect on the development of dermatitis. We do not know the reason but side effects such as alopecia and atrophy of the skin may have masked the anti-dermatitis activity, since the steroid ointments showed an inhibitory effect on the changes observed by histopathological studies.

Plasma IgE level was elevated even in the younger mice, and the increment coincided with the development of dermatitis in older mice. Although the level of plasma IgE clearly declined in the FK506- and steroids-treated animals, the mechanisms by which this was achieved was not clarified. The activity of inflammatory cells such as mast cells and Langerhans cells may be suppressed by lowering plasma IgE level. In fact immunostaining of IgE in the dermis was decreased in both the FK506- and steroids-treated NC mice, even though this may be related with the decrease of mast cell numbers. Plasma IgG_1 level was also decreased, but IgG_{2a} level was not affected. Since it was reported that IgE and IgG_1 are regulated by IL-4 from T helper type 2 (Th2) cells (19) and IgG_{2a} by IFN-γ from Th1 cells (20) in mice, FK506 might suppress the Th2 cells selectively. Since the intensity of immunostaining of IL-2 and IFN-γ was weak, Th2 cells might be especially augmented in NC mice. On the other hand, mast cells are reported to produce other cytokines including IL-4 (21, 22). It was also reported that mast cells in the skin of NC mice produced IL-4 (4). FK506 might suppress the production of IL-4 from the mast cells. This should be made clear by in vitro studies.

FK506 has been shown to have less non-specific cytotoxic effect on stem cells than that of the steroids (9), and skin atrophy by the steroid ointments has been reported in animals and humans (23, 24). We also observed the changes in the steroid-treated rats, but not in the FK506-treated rats (25). These results suggest that the action of FK506 is specific to the inflammatory cells, and therefore, the clinical side effects of FK506 will be less than those of steroids in atopic dermatitis patients.

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Attachment 2

Original Paper

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Atopic Dermatitis-Like Skin Lesions Induced by Topical Application of Mite Antigens in NC/Nga Mice

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Key Words
Allergy · Animal model · Mite · Th2 response · Dermatitis

Abstract
Background: Atopic dermatitis (AD) is a chronic relapsing inflammation usually observed in patients with an individual or a familial history of atopic diseases, precipitated by environmental factors including mite antigens (Ag). However, the exact etiology of AD is unclear. To further explore the pathogenesis and treatment of AD, a suitable animal model is necessary. In this study, we developed a new animal model of AD induced by mite Ag in NC/Nga mice. ***Methods:*** We injected the extracts of mite Ag intradermally at the ventral side of the ear of SPF NC/Nga mice on days 0, 2, 4, 7, 9, 11, 14 and 16, and measured the clinical symptoms and the ear thickness. On day 18, we collected blood and submandibular lymph nodes (LN) of the immunized ear to perform a histochemical analysis, and to measure the plasma immunoglobulins and cytokines. ***Results:*** The NC/Nga mice immunized with mite Ag suffered from AD-like skin lesions including erythema followed by edema, excoriation and scaling. The histological and immunohistochemical examinations of the affected skin showed epidermal hyperplasia with hyperkeratosis, severe infiltration of CD4+ T lymphocytes, eosinophils and macrophages, and degranulation of mast cells. The total plasma IgE level was markedly elevated in mite Ag-treated mice. LN cells of mice immunized with mite Ag synthesized IgE in an Ag-dependent manner and secreted interleukin-4 (IL-4) and IL-5 but not interferon-γ. ***Conclusions:*** NC/Nga mice treated with mite Ag manifest clinical and immunological aspects similar to patients with AD, suggesting that this model is suitable for exploring the pathogenesis of human AD.

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Introduction

Atopic dermatitis (AD) is one of the most common skin diseases in patients with a personal or a family history of atopic disorders [1], and its prevalence is still increasing. Patients with AD frequently show elevated immunoglobulin E (IgE) levels against many kinds of allergens [2]. Epidemiological studies [3, 4] suggest that environmental factors including mite antigens (Ag) [5, 6],

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air pollution [7] and mental stress might contribute to the onset or progression of AD [8]. Mite Ag have been focused on because of their high potency as allergens. More than 90% of patients with AD are positive for the IgE radioallergosorbent test (IgE-RAST) against mite Ag [9], and clinical symptoms of AD improved after mites had been removed [10, 11]. *Dermatophagoides pteronyssinus* (Dp), a representative allergen of human AD, is involved in the pathogenesis of AD [12]. Although mite Ag are major allergens in human AD, there is no suitable animal model for investigating the importance of mite Ag in AD.

It is known that T helper (Th) cells can be grouped into two functionally distinct subsets, Th1 and Th2, characterized by the different effector cytokines that they secrete. Th1 cells produce IL-2 and IFN-γ, whereas Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 which enhance humoral immunity. Overexpression of IL-4 and IL-5 was observed in the affected skin sites of AD [13, 14], and IFN-γ production by T cells of the patients with AD was lower than that by T cells of healthy donors [15–17], suggesting the polarization of Th2 in patients with AD. Although Th2 seems to contribute to the pathogenesis of AD, the differentiation process of Th2 in patients with AD is not completely understood. Therefore, developing an animal model showing a Th2-dominant immune reaction would help to clarify the pathogenesis of AD.

Matsuda and his colleagues [18–21] proposed that NC/Nga mice might become an excellent animal model for human AD. NC/Nga mice were established as an inbred strain in 1957 [22, 23] and have the following biological characteristics: liver and kidney esterase like a DBA/2 strain, high susceptibility to X-irradiation and high susceptibility to anaphylactic shock. NC/Nga mice raised in air-uncontrolled conventional circumstances spontaneously suffer from AD-like skin lesions with a marked elevation in plasma levels of total IgE, whereas NC/Nga maintained in specific pathogen-free (SPF) conditions do not show any clinical symptoms. However, a variety of Ag can be involved in the development of AD-like skin lesions in NC/Nga, which makes it difficult to analyze the pathogenesis of AD-like skin lesions. To further investigate the pathophysiology of AD, a specific Ag-induced model is necessary.

Environmental factors are thought to trigger the skin lesions of AD since NC/Nga mice grown in SPF circumstances showed neither clinical signs nor IgE hyperproduction. The mice maintained under conventional conditions are often infected with fur mites, and the infection can provoke dermatitis in NC/Nga mice [24]. However, it has not been clearly understood whether mite Ag causes AD-like skin lesion in NC/Nga mice without other environmental factors. Excluding the involvement of environmental factors is significant in order to analyze the pathogenesis of AD-like dermatitis in NC/Nga mice, and SPF conditions are preferable for analyzing the pathogenicity of mite Ag.

In this study, a local injection of Dp, a major allergen, could induce AD-like skin lesions in NC/Nga mice in SPF conditions. Lymphocytes in lymph nodes (LN) of Dp-injected mice produced IL-4 and IL-5, but not IFN-γ, and produced anti-Dp-specific IgG and IgE in response to Dp. These results demonstrated that Dp could evoke an Ag-specific Th2 immune response in NC/Nga mice and suggest that Dp-injected NC/Nga mice are a useful model for investigating the pathogenesis of AD.

Material and Methods

Mice

Six-week-old SPF NC/Nga or BALB/c female mice were purchased from Charles River Japan (Osaka, Japan) or Fujisawa Technical Services (Osaka, Japan), and maintained in SPF conditions for more than 1 week before use. All conditions related to keeping and handling the animals were approved by the Japanese Society of Laboratory Animals and implemented according to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Cytokines and Antibodies

Monoclonal antibodies (Abs) for mouse CD4 (PharMingen; San Diego, Calif., USA) and mouse F4/80 (Serotec, Oxford, UK) were used for immunohistochemistry. Fluorescein isothiocyanate (FITC)-conjugated monoclonal Abs (mAbs) for mouse T cell receptor (TCR) αβ, CD4, CD8α, CD69, Mac-1, B220 and IFN-γ, and phycoerythrin (PE)-conjugated mAb for mouse IL-4 were purchased from PharMingen. Recombinant IL-6 was purchased from PharMingen. For enzyme-linked immunosorbent assay (ELISA), mAbs for mouse IL-2 (JES6-1A12), IL-4 (BVD4-1D11), IL-5 (TRFK5) and IFN-γ (R4-6A2) as capture Abs, and biotin-conjugated mAbs for IL-2 (JES6-5H4), IL-4 (BVD6-24G2), IL-5 (TRFK4) and IFN-γ (XMG1.2) as detection Abs were also purchased from PharMingen.

Repeat Application of Mite Ag

NC/Nga mice were injected intradermally with saline or 5 μg of Dp extract (LSL, Japan) dissolved in saline on the ventral side of their right ears on days 0, 2, 4, 7, 9, 11, 14 and 16. The ear thickness of the ear injected with Ag was measured with an ear thickness gauge (OZAKI MFG, Osaka, Japan) 24 h after each intradermal injection. On day 18, blood was collected and the plasma sample was stored at −20°C until quantitative analysis for Igs.

Cytokines and Igs Production by LN Cells

Submandibular LN were removed in sterile conditions from Dp-injected and age-matched nontreated mice on day 18, and single-cell suspensions were prepared in RPMI 1640 (Life Technologies; Grand

Island, N.Y., USA) supplemented with 10% FCS (Hyclone; Logan, Utah, USA), 10^{-4} *M* 2-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were incubated in 24-well culture plates (Sumitomo Bakelite; Tokyo, Japan) at a concentration of 5×10^6 cells/ml with 10 μg/ml of Dp, 1 μg/ml of concanavalin A (Con A; WAKO Chemical Corp., Osaka, Japan) or 100 U/ml of recombinant IL-6 at 37°C under 5% CO_2. The culture supernatants were collected after 24 h and 7 days to determine cytokine and Ig production, respectively.

ELISA

IgG titer in plasma specific to Dp were measured by ELISA using Dp and anti-mouse IgG mAb. Briefly, 96-well immunoplates (Sumitomo Bakelite) were coated with 50 μl of Dp (50 μg/ml) or saline (PBS) for negative control and incubated for 1 h at 37°C. After washing 3 times with PBS containing 0.05% Tween 20, the plates were blocked with PBS supplemented with 0.5% BSA for 30 min at room temperature. After washing, 100 μl of standards and samples were added to the wells and incubated for 1 h at room temperature. Then 50 μl of peroxidase-conjugated anti-mouse IgG (Zymed; San Francisco, Calif., USA) was added and they were further incubated for 30 min at room temperature. The plates were washed and substrate (*o*-phenylenediamine tablet; Sigma, St. Louis, Mo., USA) and H_2O_2 mixed according to the manufacturer's instructions) were added. The absorbance at 492 nm wavelength was measured which SPECTRA MAX 250 (WAKO, Osaka, Japan) after stopping the reaction with H_2SO_4. Total IgE levels were measured with sandwich ELISA using two kinds of rat anti-mouse IgE mAb. Immunoplates (Sumitomo Bakelite) were coated with 50 μl of mAb to mouse IgE (10 μg/ml; YAMASA, Chiba, Japan) and incubated for 1 h at 37°C. Plates were washed and blocked. Collected samples or standard mouse IgE (YAMASA) were added to the wells and incubated for 1 h at room temperature. After washing, 50 μl biotinylated mAb for mouse IgE (YAMASA) was added to the wells and incubated for 1 h at room temperature. Two hundred microliters of peroxidase-conjugated streptavidin (Zymed) was added and incubated for 1 h at 37°C. After washing, *o*-phenylenediamine was added and the reaction was stopped with the addition of H_2SO_4. Then the absorbance at 492 nm wavelength was measured. Concentrations of IL-2, IL-4, IL-5 and IFN-γ in culture supernatants were also measured with sandwich ELISA similar to that of total IgE. Immunoplates (Nunc Roskilde, Denmark) and a TMB Peroxidase EIA Kit (Bio-Rad Laboratories, Hercules, Calif., USA) as a substrate were provided and the absorbance at 450 nm wavelength was measured after stopping the reaction with H_2SO_4. The sensitivities of IL-2, IL-4, IL-5 and IFN-γ assays were 3.125, 3.906, 3.906 and 50 pg/ml, respectively.

Flow-Cytometric Analysis

To examine the cell populations in LN, flow-cytometric analyses were performed. Freshly isolated LN cells were incubated with mAbs for TCRαβ, CD4, CD8α, CD69, Mac-1 or B220 for 30 min at 4°C and analyzed by fluorescence-activated cell sorter (FACScan; Becton Dickinson, Bedford, Mass., USA). To examine the populations of IL-4 and IFN-γ producing cells in submandibular LN, cell preparations were incubated with Dp (10 μg/ml) for 19 h and treated with CytoStain™ Kits (PharMingen) for an additional 5 h. Then the cells were harvested and stained with FITC-conjugated anti-mouse IFN-γ and PE-conjugated anti-mouse IL-4 Ab, and analyzed.

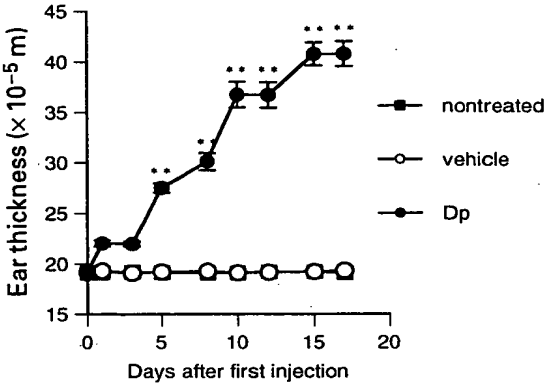


Fig. 1. Ear thickness and clinical features of the ear of mice injected with Dp or vehicle and age-matched nontreated mice. NC/Nga mice were injected with Dp (●) or vehicle (○) on day 0, 2, 4, 7, 9, 11, 14 and 16. Ear thickness was evaluated by a thickness gauge 24 h after each injection. That of age-matched nontreated mice (■) was evaluated at the same time when Dp and vehicle (saline-injected) mice were done. Each symbol represents the mean ± SE of 10 mice. ** $p < 0.01$, when compared with the vehicle group.

Histological Analysis

Right ears of Dp-injected and nontreated mice were removed 2 days after the last injection of Dp, and one part of the ear was fixed in 10% phosphate-buffered formalin (pH 7.2), embedded in paraffin, cut in 3 μm, and stained with hematoxylin and eosin or toluidine blue (pH 4.0). The other part of the ear was embedded in OCT compound (Miles; Elkhard, Ind., USA) and snap-frozen in dry-ice ethanol for immunohistochemistry. Frozen sections cut in 5 μm were fixed in cold acetone. After blockage of endogenous peroxidase, mAb for mouse CD4 or F4/80 was applied overnight at 4°C, and then goat anti-rat IgG conjugated with peroxidase (Jackson, West Grove, Pa., USA) was overlayed. The enzyme reaction was visualized with 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, Wisc., USA).

Statistical Analysis

Data were expressed as mean ± SE. Dunnet's multiple comparison was performed for statistical analysis and $p < 0.05$ was taken as the level of significance.

Results

Development of AD-Like Skin Lesions

Since Dp is one of the major allergens involved in the pathogenesis of human AD, we examined whether mite Ag could induce AD-like disease in NC/Nga mice raised in SPF conditions. We injected the extracts of Dp intradermally at the ventral site of the ear of SPF NC/Nga mice

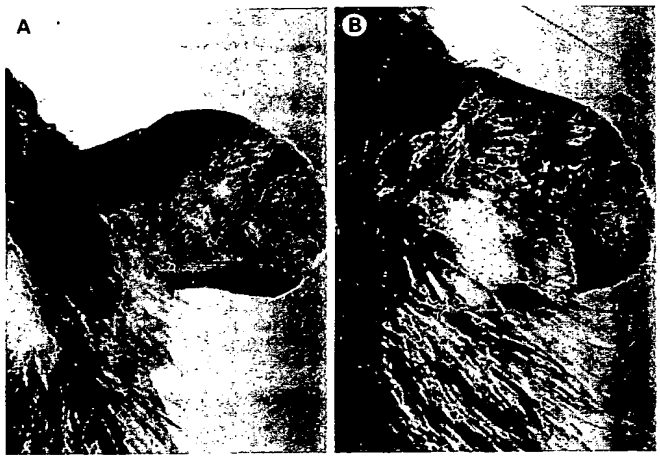


Fig. 2. Clinical feature of the ears of NC/Nga mice on day 18. Erythema, edema, excoriation and scaling were noticed in the ear injected with Dp (**B**), but not with vehicle (**A**).

and observed ear thickness and the clinical skin severity 24 h after every application of Ag. From day 5–8, the ear thickness was definitely increased (fig. 1). The ear thickness and the severity of the skin lesions were increased with further Ag injections until day 18. Erythema, edema, excoriation and scaling were observed at the dorsal site of the ears (fig. 2). Itching was also observed. The mice injected with vehicle showed neither skin lesions nor ear thickening throughout the application (fig. 1). Neither increased ear thickness nor skin lesions were observed in BALB/c mice, in which Dp had been used in the same way as in NC/Nga mice (data not shown).

Histopathological Analysis of the Skin Lesion

The skin lesions were examined histopathologically on day 18. Epidermal hyperplasia with areas of parakeratosis and severe infiltrations of eosinophils and a small number of mononuclear cells in the dermis of ears injected with Dp were found (fig. 3B, table 1). We also observed an increased number of mast cells (table 1) and most of the mast cells were degranulated (fig. 3C, D) in the skin lesions but not in the nontreated ear. A great number of CD4+ T lymphocytes and F4/80+ macrophages were identified in the dermis of the ear injected with Dp by immunohistochemical staining, whereas neither cell was found in the ear of nontreated mice (fig. 3F, H, table 1).

Anti-Dp-Specific IgG Titer and Total IgE Levels in Plasma

IgG and IgE are considered to be associated with symptoms of AD, and plasma levels of anti-Dp-specific IgG and total IgE in NC/Nga mice on day 18 were quantitated with ELISAs. The total IgE level in Dp-injected mice was higher than those in nontreated mice (fig. 4B). Anti-Dp IgG were detected in Dp-injected mice, but not in nontreated mice (fig. 4A).

In vitro Ig Production by Dp

At first, we investigated the cell number and cell population of drainage LN and spleen of Dp-injected NC/Nga mice, and compared them with those of nontreated mice. The cell number of submandibular LN of Dp-injected mice was dramatically elevated when compared to those of nontreated mice (fig. 5A). However, the splenic cell number of Dp-injected mice was the same as that of non-

Table 1. Numbers of eosinophils and mast cells in dorsal skin of vehicle and Dp-injected NC/Nga mice

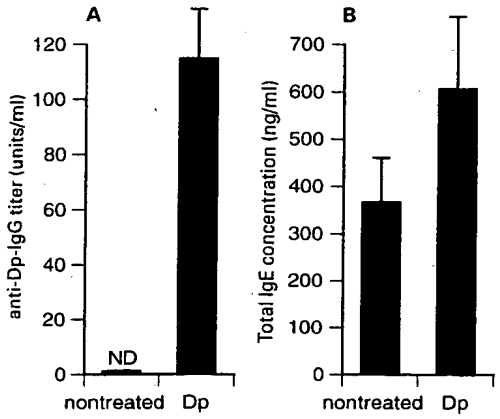
Skin	Number of cells/5 fields				
	eosinophils	mast cells			T cells CD4+
		granulated	mildly degranulated	severely degranulated	
Nontreated	0	36.3±3.6	0	0	3.8±0.7
Dp	252±21	18.8±2.8	24.0±3.0	43.1±3.9	60.0±6.4

The numbers of eosinophils, the degranulation of mast cells, and CD4+ cells in dorsal skin of vehicle and Dp-injected NC/Nga mice. Hematoxylin and eosin staining for the calculation of eosinophils, toluidine blue staining for mast cells and immunostaining for CD4+ cells were done as described in Materials and Methods. Cells between epithelium and panniculus carnosus were counted under a microscope at a magnification of ×400 and were expressed as the total number of cells in 5 fields. Each value was obtained from 6 mice per group (mean ± SE).

Fig. 3. Histological analysis of the nontreated and Dp-injected ears on day 18. Hematoxylin and eosin staining preparation demonstrated that hyperplasia of the epidermis and infiltration of eosinophils (insert) in the dermis were pronounced in Dp-injected mice (**B**), but not in nontreated mice (**A**). It was shown in toluidine blue-staining preparation that mast cells in Dp-injected mice (**D**) were degranulated, whereas those in nontreated mice (**C**) were not. Immunostaining for CD4 of nontreated (**E**) and Dp-injected mice (**F**), and for F4/80 of nontreated (**G**) and Dp-injected mice (**H**) demonstrated that infiltrations of CD4+ T lymphocytes and F4/80+ macrophages were observed in Dp-injected mice.

treated mice (fig. 5B). To determine the population that had proliferated in response to Dp, freshly isolated LN cells were analyzed with a flow cytometry. B220+ cells and CD69+ T cells in LN of Dp-injected mice were increased (table 2).

Next, to confirm the contribution of Dp in this model, the response of drainage LN cells of Dp-injected mice to Dp was analyzed and compared with those of nontreated mice. LN cells of Dp-injected and nontreated mice were cultured with Dp or IL-6, and anti-Dp-specific IgG titer and IgE concentration in the culture supernatant were examined. When LN cells were incubated with Dp, LN cells of Dp-injected mice produced anti-Dp-specific IgG and IgE (fig. 6), whereas those of nontreated mice could produce neither Igs. Splenic cells did not synthesize Igs when stimulated with Dp (data not shown).



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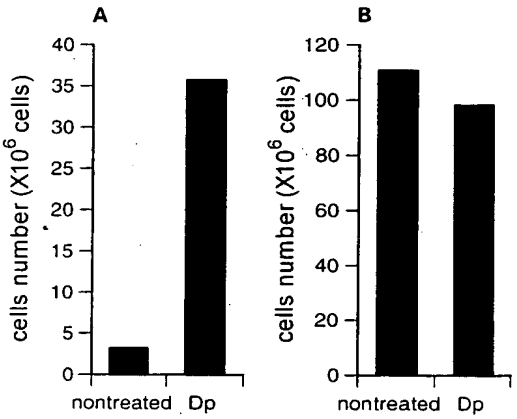

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Fig. 4. Plasma levels of anti-Dp-specific IgG and total IgE. Blood was collected on day 18 from the retroorbital plexus and diluted 4 times with saline, and plasma samples were obtained. Anti-Dp-specific IgG titer (**A**) and total IgE level (**B**) were measured with ELISA as described in Materials and Methods. Each point represents the mean ± SE of 7 mice. ND = Not detected.

Fig. 5. Cell number of submandibular LN and spleen of nontreated and Dp-injected mice. LN and spleens of nontreated and Dp-injected mice were collected and homogenated to make a single-cell suspension. The cell numbers of freshly isolated LN cells (**A**) or splenic cells (**B**) were counted with 0.25% trypan blue solution. These data represent six separate experiments.

Fig. 6. In vitro Ig production by LN cells in response to Dp. 5×10^6 cells isolated from submandibular LN of nontreated and Dp-injected mice were incubated in 24-well culture plates with Dp (10 μg/ml) or IL-6 (100 U/ml) for 7 days. Anti-Dp-specific IgG titer (**A**) and total IgE level (**B**) in the culture supernatants were measured with ELISA. Ig production by LN cells of nontreated mice (□) and Dp-injected mice (■) is shown. These data represent six separate experiments performed in duplicate. ND = Not detected.

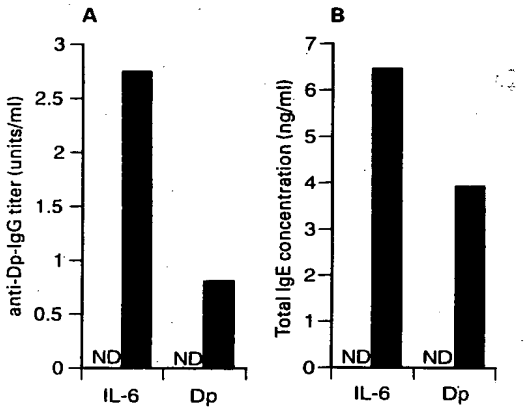

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Table 2. Population in LN cells (%)

	TCRαβ	CD4	CD8α	CD69	Mac-1	B220
Nontreated	60.95	38.82	18.39	10.73	8.83	35.28
Dp	45.53	29.81	15.77	18.21	0.77	55.16

FACS analysis in LN cells. LN cells were freshly obtained on day 18. The cells were stained with either FITC-conjugated mAbs for mouse TCRαβ, CD4, CD8α, CD69, Mac-1 or B220. The cell preparations were analyzed by a flow cytometer. The percentage of positive cells was calculated from the control. The data represent three experiments.

Table 3. Th1/Th2 population in LN (%)

Cell type	Nontreated	Dp-injected
IL-4– IFN-γ–	98.84±0.68	90.1±4.26
IL-4+ IFN-γ–	0.77±0.64	8.38±3.14
IL-4– IFN-γ+	0.14±0.03	0.50±0.30
IL-4+ IFN-γ+	0.19±0.11	1.02±0.81

Values represent mean ± SE. LN cells were isolated from Dp-injected or nontreated mice on day 18, and stimulated with Dp (10 μg/ml) for 19 h. Then cells were harvested and stained with FITC-conjugated anti-mouse IFN-γ mAb and PE-conjugated anti-mouse IL-4 mAb, and analyzed by FACScan.

Th2 Responses in LN Cells of Dp-Injected Mice

The elevation of the plasma IgE level is induced by Th2 differentiation. Therefore, we investigated the population of IL-4-producing cells in LN (table 3). Submandibular LN cells were incubated with Dp for 19 h, and intracellular IL-4 and IFN-γ were investigated with FACScan analysis. The IL-4+ IFNγ– Th2 population was greater than the IL-4– IFN-γ+ Th1 population in LN cells of Dp-injected mice. The population of both Th1 and Th2 was small in LN of nontreated mice in response to Dp.

Next, IL-2, IL-4, IL-5 and IFN-γ synthesis by LN cells of Dp-injected or nontreated mice was examined (fig. 7). When LN cells of nontreated mice were incubated with Dp for 24 h, IL-4, IL-5 or IFN-γ in the culture supernatant were below the detection level of ELISA. IL-4 and IL-5 in the culture supernatant were below the detection level of ELISA. IL-4 and IL-5 in the culture supernatant of LN cells isolated from Dp-injected mice were detected but not IFN-γ, despite there being a detectable level of IFN-γ in the culture supernatant of LN cells of Dp-injected mice stimulated with Con A. Only IL-2 was detectable in the culture supernatant of LN cells isolated from nontreated mice stimulated with Con A.

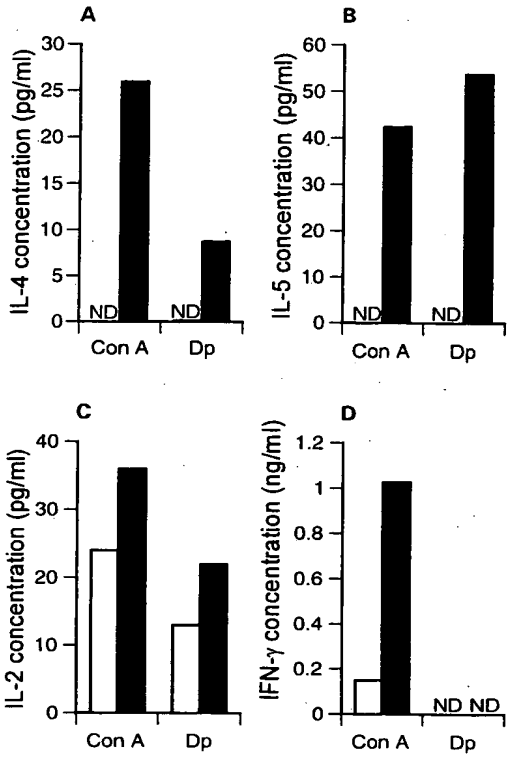

Fig. 7. Cytokine production by LN cells in response to Dp. LN cells isolated from mice injected with Dp (■) or vehicle (□) were stimulated with Dp (10 μg/ml) or Con A (1 μg/ml) for 24 h. Concentrations of IL-4 (**A**), IL-5 (**B**), IL-2 (**C**) and IFN-γ (**D**) in culture supernatants were measured with ELISA as described in Materials and Methods. These data represent three separate experiments performed in duplicate. ND = Not detected.

Discussion

The mice injected with Dp manifested erythema, edema, excoriation and scaling only in the injected sites and also showed an increased level of plasma IgE. Histopathologically, the degranulation of mast cells, and infiltration of eosinophils, CD4+ T cells and macrophages to dermis were noticed in the skin lesion. All these findings are typical symptoms observed in patients with AD, indicating the relationship of IgE, mast cells, eosinophils, CD4+ T cells and macrophages to the pathogenesis of AD-like skin lesions. Thus, this model has the same characteristics of human AD and is useful for clarifying the pathogenesis of AD.

In this study, we have demonstrated that intradermal injections of mite Ag extracts, a major Ag participating in the development of AD, could induce AD-like skin lesions in NC/Nga mice. Matsuda and his colleagues [18–21] demonstrated that NC/Nga mice suffered AD-like skin lesions when they were maintained in air-uncontrolled conventional circumstances, and they suggest that several environmental factors may be involved in the pathogenesis of the dermatitis. In patients with AD, mite Ag is one of the major environmental factors, and it is important to induce AD-like skin lesions by mite Ag for investigating the significance of mite Ag to human AD. We induced AD-like dermatitis in NC/Nga mice only with mite Ag extracts without other environmental factors, and this model might show further information relevant for the pathogenesis of human AD.

Our data indicate that mite Ag induce Th2 reactions in NC/Nga mice. The percentage of IL-4+ IFN-γ– cells in the LN cells stimulated with Dp increased and the cells generated IL-4 and IL-5, but not IFN-γ, suggesting Ag-specific IgG and IgE production and the infiltration of eosinophils. Mite Ag are reported to induce Th2 cytokines and responses [25–27]. IL-4 is the principal factor for IgE synthesis, and IL-5 is also the key factor for the activation/

proliferation of eosinophils [14, 28, 29]. Th2 responses are often noticed in patients with AD, and we can observe Th2 responses in this model which are the same as in patients with AD [27, 30, 31]. Therefore a further investigation of this model will provide us with useful information about the mechanism of human AD.

We have demonstrated that mite Ag without adjuvants can induce AD-like dermatitis in SPF NC/Nga mice. Immunization without adjuvants does not usually provoke a strong immune reaction [32–36]. In this study, mite Ag without adjuvants generated skin lesions and a Th2 immune response in NC/Nga mice. Mite Ag could not induce severe clinical symptoms in BALB/c, suggesting that NC/Nga mice might have a genetic background making them susceptible to skin lesions in response to mite Ag like patients with AD. Recent studies have demonstrated the complex interrelationship of genetic and environmental factors that contribute to the development of human AD [37]. We demonstrated that mite Ag, which is a major environmental factor in patients with AD, could induce AD-like dermatitis in NC/Nga mice which might have some genetic background. This model might show the relationship between mite Ag and genetic background in AD-like dermatitis.

In conclusion, we have succeeded in inducing AD-like skin lesions and Th2 responses in SPF NC/Nga mice with mite Ag alone. This model is very similar to human AD, suggesting that it is suitable for clarifying the pathogenesis of AD.

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Attachment 1

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Topical Application of FK506 (Tacrolimus) Ointment Inhibits Mite Antigen-Induced Dermatitis by Local Action in NC/Nga Mice

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Key Words
FK506 · Mite antigen · T helper 2 cells · Atopic dermatitis · Lymph node · NC/Nga mice

Abstract
Background: FK506 ointment (tacrolimus ointment, protopic) is a new drug therapeutically effective for patients with atopic dermatitis (AD). However, the mechanism of action of FK506 ointment on AD is not fully understood. ***Methods:*** We examined the effect of FK506 ointment on mite antigen-induced dermatitis in NC/Nga mice. Clinical symptoms and ear thickness were recorded, and histopathological studies and in vitro analyses were performed. ***Results:*** Topical application of FK506 ointment (0.03–0.3%) suppressed the development of dermatitis. In the lesional skin, both interleukin (IL)-4 and interferon (IFN)-γ were detected, even though the IL-4+/IFN-γ– T helper 2 (Th2) population was predominant in the regional lymph nodes (LNs). Topical application of FK506 treatment reduced the elevated level of both IL-4 and IFN-γ in the skin, but did not decrease the expansion of the Th2 population in the LNs. ***Conclusions:*** Topical application of FK506 ointment suppresses dermatitis by inhibiting the activation of inflammatory cells locally, without systemic immune suppression, in this AD model.

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Introduction

Mite antigen (Ag) is a major environmental factor in atopic dermatitis (AD), with 70% of AD patients positive for skin patch tests against mite Ag [1]. Repeated environmental exposures to mite Ag are thought to maintain or exacerbate the AD symptoms, and symptoms are often improved when mites have been removed [2].

NC/Nga mice are a good model for AD, with environmental factors producing dermatitis in them. NC/Nga mice in conventional conditions, without air control, suffer from AD-like skin lesions, but these mice do not show any clinical symptoms under specific pathogen-free (SPF) conditions [3]. A recent study demonstrated that infection with fur mites could provoke dermatitis in NC/Kuj mice, a substrain of NC/Nga mice [4]. To analyze the pathogenesis of AD, we previously established a mite Ag-induced AD model with NC/Nga mice under SPF conditions [5].

Intracellular adhesion molecule-1 (ICAM-1, CD54) is expressed on Ag-presenting cells, keratinocytes and endothelial cells, and is required for infiltration and activation of leukocytes [6]. Vascular cell adhesion molecule-1 (VCAM-1, CD105) is also expressed on endothelial cells, and is required for infiltration of eosinophils. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β and interferon (IFN)-γ induce ICAM-1 expression, and TNF-α, IL-1β and IL-4 produce VCAM-1 expression on endothelial cells. Our mite Ag-induced AD model with NC/Nga mice shows severe infiltration of inflammatory cells, especially

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infiltration of eosinophils. Expression of the adhesion molecules may explain the severe inflammation.

FK506, a hydrophobic macrolide lactone produced by *Streptomyces tsukubaensis*, exerts profound immunosuppressive effects in vitro and in vivo [7, 8]. FK506-FKBP12 complex suppresses the activation of T cells by inhibiting calcineurin phosphatase activity. Topical application of FK506 ointment exerts good therapeutic efficacy in atopic eczema [9]. FK506 ointment penetrates through skin [10], and clinical studies have shown that FK506 reduces cytokine production and infiltration of T helper (Th) cells in the skin of AD patients [11]. Treatment with FK506 ointment in the conventional NC/Nga model also produces a decreased level of clinical signs and the reduction of Th2 cytokines in the skin [12]. However, whether FK506 ointment affects systemic immune modulation or acts only locally in the treated skin is still unclear.

The aim of this study was to analyze the mechanisms of topical FK506 in vivo. For this purpose, we analyzed the effect of FK506 ointment on cytokine production and adhesion molecule and T cell activation in the skin, then compared these results to those in the regional lymph nodes (LNs) in NC/Nga mice.

Materials and Methods

Mice

SPF NC/Nga female mice (15–20 g) at the age of 6 or 7 weeks were purchased from Charles River Japan (Yokohama, Japan). The animals were maintained in groups of 4 for at least 7 days on a 12-hour light-dark cycle (lights on from 07.00 to 19.00 h) in a controlled temperature (23 ± 1 °C) and humidity (55 ± 5%) environment. The mice were given standard laboratory food and tap water ad libitum before the experiment. All conditions and handling of the animals were approved by the Japanese Society of Laboratory Animals. In addition, all animal experimental procedures were performed according to the guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical Co. Ltd.

Reagents

FK506 ointment (0.03–0.3%) and placebo were obtained from Fujisawa Pharmaceutical (Osaka, Japan). Corticosteroid ointment, betamethasone valerate (Rinderon®-V ointment, 0.12%), was purchased from Shionogi (Osaka, Japan).

Cytokines and Antibodies

Monoclonal antibodies (mAbs) for mouse CD4 (PharMingen, San Diego, Calif., USA), IL-4 (PharMingen), IFN-γ (PharMingen), ICAM-1 (Seikagaku Corp., Tokyo, Japan), VCAM-1 (PharMingen), TNF-α (PharMingen) and IL-1β (R & D, Minneapolis, Minn., USA; Genzyme, Cambridge, Mass., USA) were used for immunohistochemistry. Abs were diluted to 0.5, 25, 10, 5, 5, 10 and 1 μg/ml, respectively, and used for immunostaining. Fluorescein isothiocyanate (FITC)-conjugated mAbs for mouse T cell receptor (TCR) αβ, CD4, CD8α, CD69, Mac-1 (CD11b), B220 (CD45R) and IFN-γ, and phycoerythrin (PE)-conjugated mAb for mouse IL-4 were purchased from PharMingen. Isotypes of both FITC-conjugated rat IgG1 and PE-conjugated rat IgG1 as negative controls were also obtained from PharMingen. All Abs were prepared for flow cytometric analysis at a concentration of 20 μg/ml.

Repeat Application of Mite Ag

Mice were immunized as described previously [5]. Briefly, NC/Nga mice were injected intradermally with saline or 5 μg/10 μl of *Dermatophagoides pteronyssinus (Dp)* extract (Cosmo Bio LSL, Tokyo, Japan) dissolved in saline, on the ventral side of their right ear on days 0, 2, 4, 7, 9, 11, 14 and 16. 10 μl of drugs were applied onto the dorsal side of the *Dp*-treated ears 3 h before and after *Dp* treatment. The thickness of the ear injected with *Dp* was measured by an ear thickness gauge (Ozaki Mfg, Osaka, Japan) before the first injection and 24 h after each intradermal injection.

Flow Cytometric Analysis

Flow cytometric analysis was performed as described previously [5]. Briefly, freshly isolated LN cells were incubated with mAbs for TCRαβ, CD4, CD8α, CD69, Mac-1 or B220 for 30 min at 4 °C and analyzed by flow cytometry (FACScan, Becton Dickinson, Bedford, Mass., USA). To examine the populations of IL-4- and IFN-γ-producing cells in regional LNs, cell preparations were incubated with *Dp* (10 μg/ml) for 19 h and treated with a CytoStain™ Kit (PharMingen). First, cells were treated with GolgiStop™ (contains monensin) for 5 h. After removing the culture medium, cells were fixed and permeabilized with Cytofix/Cytoperm™ solution (includes formaldehyde) for 20 min at 4 °C. Cells were washed and resuspended with Perm/Wash™ solution (includes saponin). Then, cells were harvested and stained with FITC-conjugated anti-mouse IFN-γ and PE-conjugated anti-mouse IL-4 Ab for 30 min at 4 °C. After washing twice with Perm/Wash solution, cells were suspended in staining buffer, then analyzed by flow cytometry.

Histological Analysis

Histological analysis was performed as described previously [5]. Briefly, right ears of *Dp*-injected and sham-injected mice were removed 2 days after the last injection of *Dp*. A part of the ear was fixed in 10% phosphate-buffered formalin (pH 7.2) and embedded in paraffin, and 3-μm sections were cut. Then those samples were stained with toluidine blue (pH 4.0) for detection of mast cells or congo red for eosinophils. The other part of the ear was embedded in OCT compound (Miles, Elkhard, Ind., USA) and snap frozen in dry ice ethanol for immunohistochemistry. Frozen 5-μm sections were fixed in cold acetone. After blocking of endogenous peroxidase, mAb for mouse CD4, IL-4, IFN-γ, ICAM-1, VCAM-1, TNF-α or IL-1β was applied overnight at 4 °C, and then goat anti-rat IgG conjugated with peroxidase (Jackson, West Grove, Pa., USA) was overlayed. Visualization of the enzyme reaction was performed with 3-amino-9-ethylcarbazole (Aldrich Chemical Company, Milwaukee, Wisc., USA).

Statistical Analysis

Data are expressed as mean ± SE. Statistical significance of differences was assessed by Dunnett's multiple comparison test following one-way analysis of variance and Student's t test for two-sample comparison. $p < 0.05$ was taken as the level of significance.

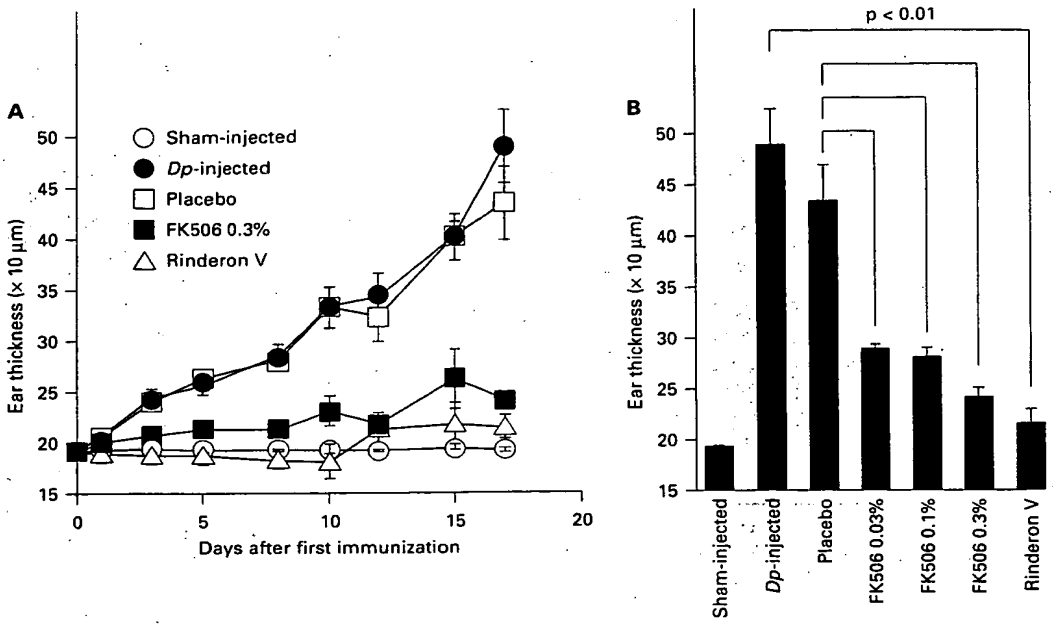


Fig. 1. FK506 ointment and steroids reduced the ear thickness of *Dp*-injected mice. NC/Nga mice were injected with *Dp* into the right ear on day 0, 2, 4, 7, 9, 11, 14 and 16. **A** Ear thickness of the right ear was evaluated by a thickness gauge 24 h after each injection. The ears of age-matched sham-injected mice were measured at the same time points as those of *Dp*-injected mice. Placebo, FK506 ointment or betamethasone valerate was applied 3 h before and after each *Dp* injection. Each data point represents the mean ± SE of 10 mice. These data are representative of 8 separate experiments. **B** Ear thickness of the right ear on day 17. Dunnett's multiple comparison was performed for statistical analysis.

Results

Effect of FK506 Ointment on Development of Dermatitis

To examine the effect of FK506 on the development of AD, we observed the clinical signs of *Dp*-injected NC/Nga mice with or without FK506 ointment treatment. *Dp* extract was injected into the ventral side of the right ears of NC/Nga mice on day 0, 2, 4, 7, 9, 11, 14 and 16, and the ear was measured 24 h after *Dp* treatment. Thickening of the ear injected with *Dp* was observed on day 5, and the thickness gradually increased until day 17 (fig. 1A). Erythema, edema, excoriation and scaling were also observed on the ear (fig. 2B). When mice were treated on the dorsal side with FK506 ointment 3 h before and after *Dp* treatment, the treated ear showed a significant decrease in thickness. On day 17, the ear thickness of *Dp*-injected mice treated with 0.03, 0.1 and 0.3% FK506 ointment was reduced by 67, 71 and 83%, respectively, compared to that of *Dp*-injected, placebo-treated mice (487.5 ± 35.6 μm; fig. 1). Only slight erythema and edema, and neither scaling nor excoriation were observed on the ears treated with FK506 ointment (fig. 2C). *Dp*-injected, betamethasone ointment-treated mice did not show any clinical symptoms or ear thickening until day 17 (fig. 1, 2D).

Histopathological Analysis of the Dermatitis

Histopathological examination of the skin lesions was performed on day 17. Congo red staining showed severe infiltration of eosinophils into the dermis of placebo-treated ears injected with *Dp*, and treatment with FK506 ointment produced a decreased eosinophil infiltration (fig. 3E, F). Most of the mast cells in the skin lesions of *Dp*-injected, placebo-treated mice were degranulated; however, degranulation of mast cells in ears treated with FK506 appeared to be relatively mild (fig. 3B, C). Treatment with steroids also demonstrated decreased eosinophil infiltration and mild degranulation of mast cells (data not shown).

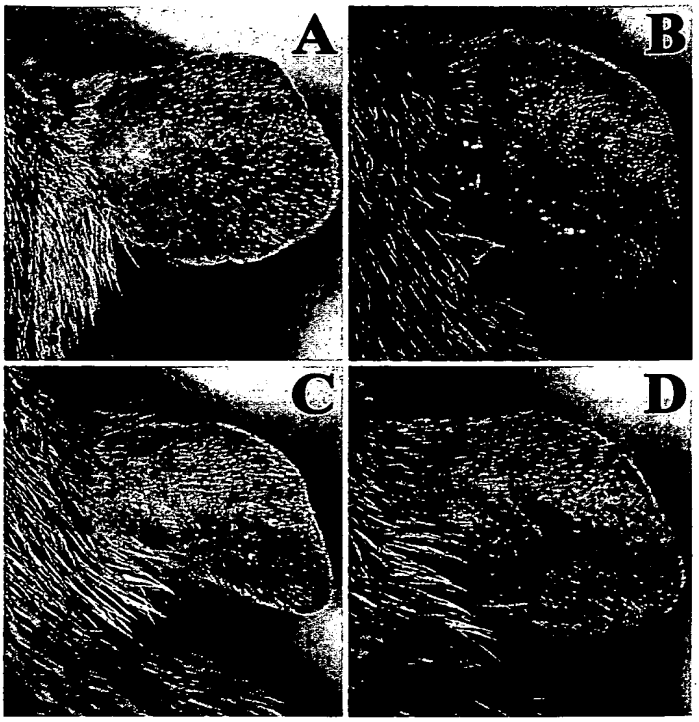
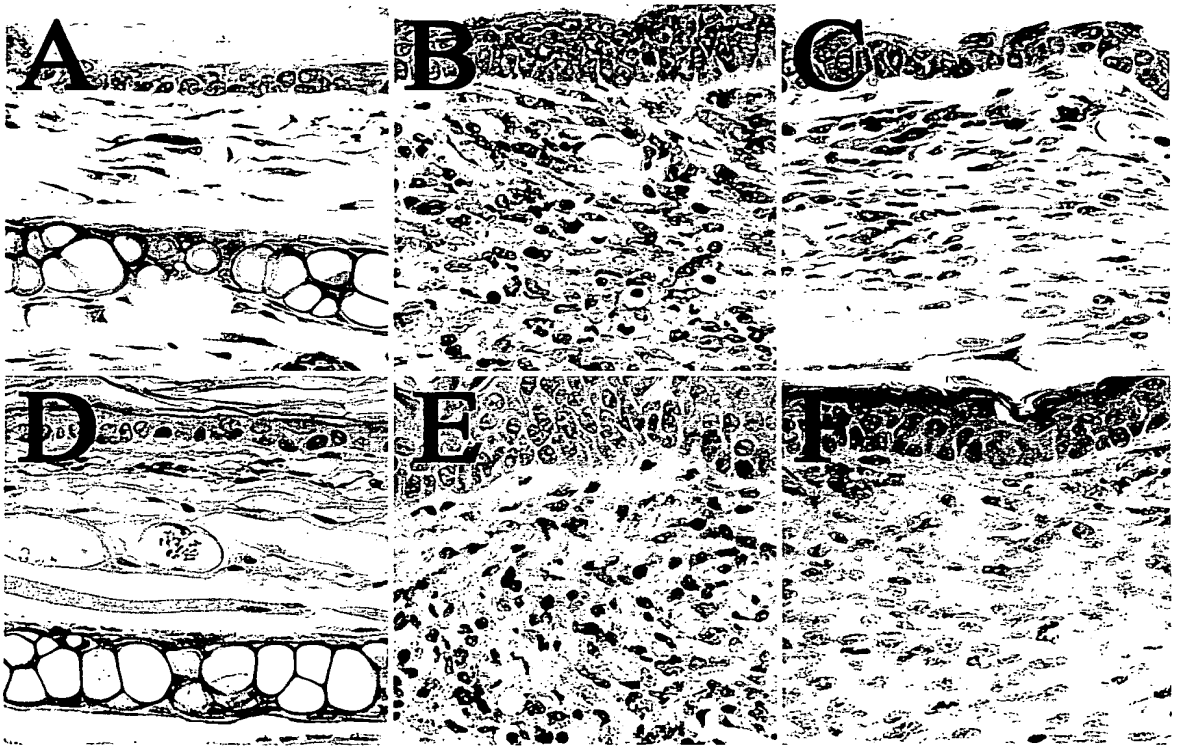


Fig. 2. Topical application of FK506 ointment or steroids improved the clinical features of dermatitis in NC/Nga mice. Shown are ears of age-matched sham-injected mice (**A**), *Dp*-injected mice (**B**), *Dp*-injected, FK506 0.3% ointment-treated mice (**C**) or *Dp*-injected, betamethasone valerate-treated mice (**D**) on day 17. NC/Nga mice were injected with *Dp* on day 0, 2, 4, 7, 9, 11, 14 and 16. FK506 ointment or steroid was applied 3 h before and after each *Dp* injection. These results are representative of three separate experiments.

Fig. 3. FK506 ointment ameliorated the degranulation of mast cells and infiltration of eosinophils in *Dp*-injected mice. Sham-injected (**A**, **D**), *Dp*-injected (**B**, **E**) and *Dp*-injected, FK506 0.3% ointment-treated (**C**, **F**) ears on day 17 were analyzed with toluidine blue staining (**A–C**) or congo red staining (**D–F**). Sections were observed at a magnification of ×400. These results are representative of three separate experiments.



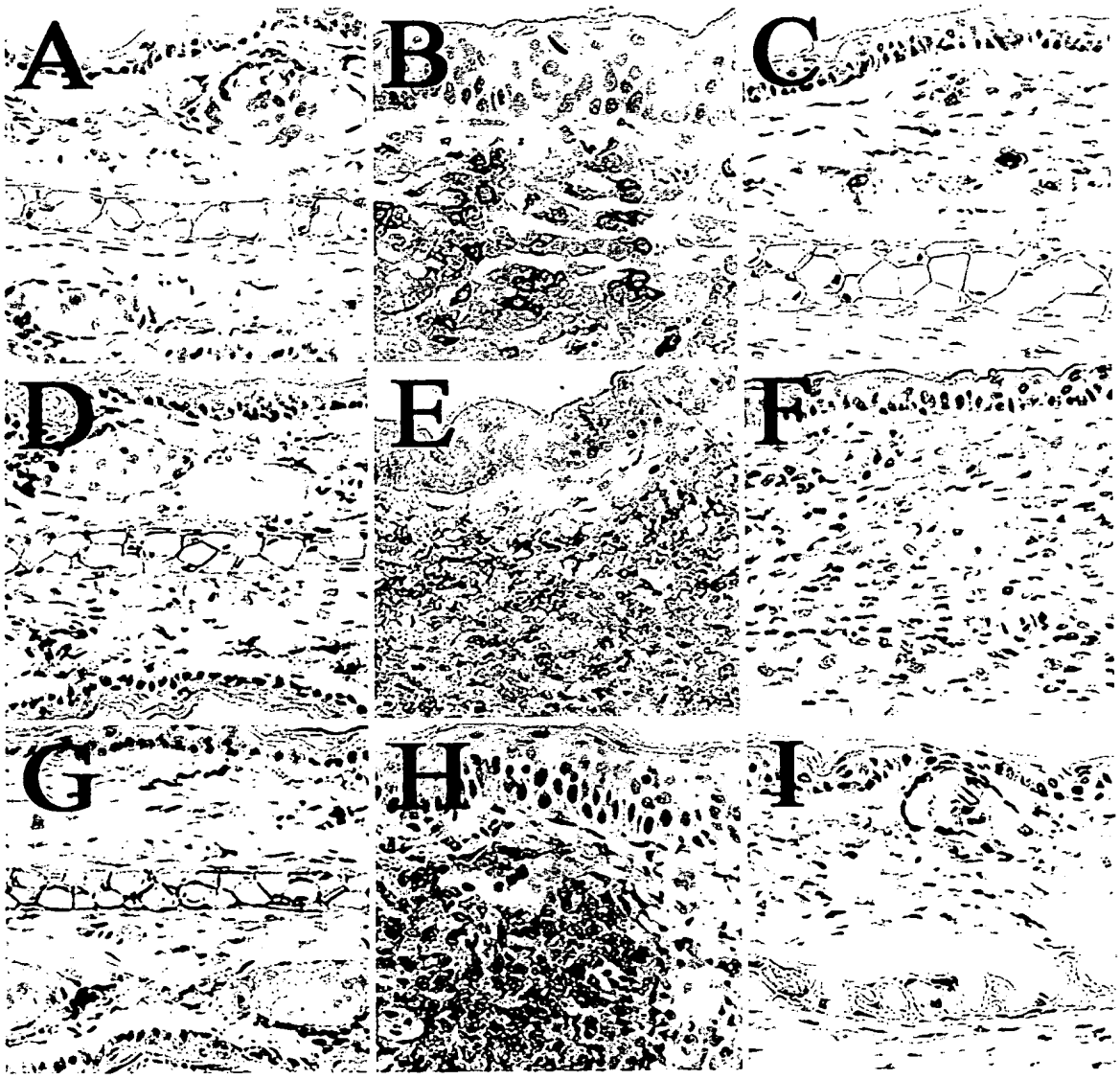


Fig. 4. FK506 ointment suppressed infiltration of Th cells and cytokine production. Sham-injected (**A**, **D**, **G**), *Dp*-injected (**B**, **E**, **H**) and *Dp*-injected, FK506 0.3% ointment-treated (**C**, **F**, **I**) ears on day 17 were stained with anti-CD4 mAb (**A–C**), anti-IL-4 mAb (**D–F**) or anti-IFN-γ mAb (**G–I**). Sections were observed at a magnification of × 200. These results are representative of three separate experiments.

Next, we assessed the lesional skin by immunohistochemical analysis. There was an increased number of CD4+ cells in skin lesions of *Dp*-injected, placebo-treated mice. FK506 ointment reduced the infiltration of CD4+ cells (fig. 4B, C). IL-4 and IFN-γ were also strongly stained in the dermis of *Dp*-injected, placebo-treated mice, but not in that of sham-injected mice. FK506 treatment reduced the staining of both IL-4 and IFN-γ (fig. 4F, I). Steroid treatment also reduced the infiltration of CD4+ cells and staining of both IL-4 and IFN-γ (data not shown).

We investigated the expression of ICAM-1 and VCAM-1 in *Dp*-injected, placebo-treated skin, and compared it to that in sham-injected skin. Both ICAM-1 and VCAM-1 were expressed in the dermis of sham-injected mice, with expression of both increased in *Dp*-injected,

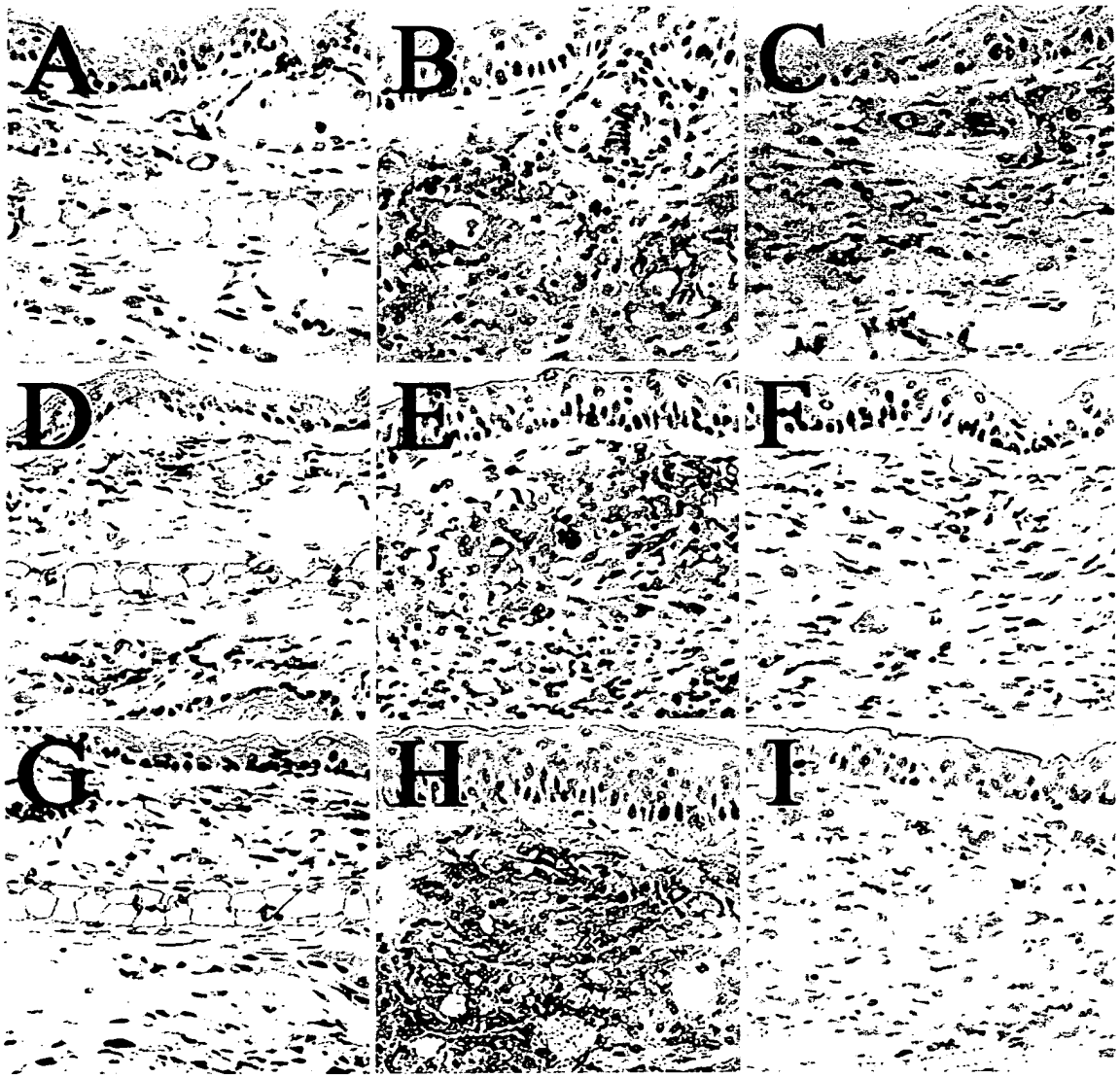


Fig. 5. FK506 ointment inhibited the expression of adhesion molecules and TNF-α. Sham-injected (**A**, **D**, **G**), *Dp*-injected (**B**, **E**, **H**) and *Dp*-injected, FK506 0.3% ointment-treated (**C**, **F**, **I**) ears on day 17 were stained with anti-ICAM-1 mAb (**A–C**), anti-VCAM-1 mAb (**D–F**) and anti-TNF-α mAb (**G–I**). Sections were observed at a magnification of × 200. These results are representative of three separate experiments.

placebo-treated mice (fig. 5B, E). Treatment with FK506 ointment reduced the expression of ICAM-1 and VCAM-1 (fig. 5C, F). TNF-α was detected in the dermis of *Dp*-injected, placebo-treated mice, but not in the dermis of sham-injected mice (fig. 5G, H). FK506 ointment reduced the expression of TNF-α in the dermis (fig. 5I).

Effect of FK506 on Reactivity of LN Cells

To determine the relationship between dermatitis and regional LNs, we investigated the cell number and cell population of regional LNs in *Dp*-injected, placebo-treated mice, and compared them to those in *Dp*-injected, FK506-treated mice. The LN cell number for *Dp*-injected, placebo-treated mice was markedly increased compared to sham-injected mice (fig. 6). The LN cell number for

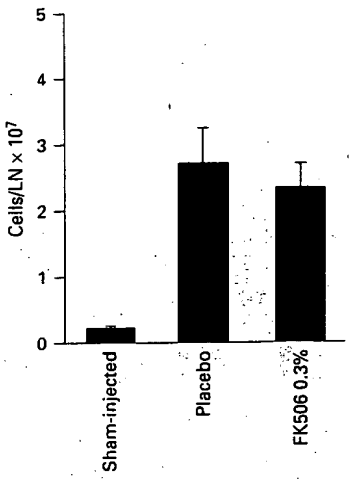


Fig. 6. FK506 ointment did not change the cell number in regional LNs. LNs of sham-injected mice, *Dp*-injected, placebo-treated (placebo) mice and *Dp*-injected, FK506 0.3% ointment-treated (FK506 0.3%) mice were collected and homogenized to make a single cell suspension. Freshly isolated LN cells were counted with 0.25% trypan blue solution. These data are representative of four separate experiments.

Table 1. Cell population in LNs

Cell type	Untreated	Placebo	FK506 0.3%
TCRαβ, %	64.24 ± 2.27	39.56 ± 4.30	37.26 ± 3.50
CD4, %	44.02 ± 3.14	27.55 ± 1.73	26.22 ± 2.68
CD8α, %	12.31 ± 6.12	18.43 ± 0.03	13.96 ± 0.41
CD69, %	7.86 ± 1.58	23.08 ± 1.87	25.54 ± 3.41
Mac-1, %	5.95 ± 2.58	1.06 ± 0.53	1.28 ± 0.59
B220, %	32.57 ± 4.48	59.59 ± 5.27	60.53 ± 6.32

LNs were freshly obtained on day 17. LNs of one group were collected as one population of LN cells. The cells were stained with FITC-conjugated mAb and cell preparations were analyzed by a flow cytometer. The percentage of positive cells was calculated from the control. Data are presented as mean ± SE of three individual experiments.

Table 2. The Th1/Th2 population in LNs

Cell type	Untreated	Placebo	FK506 0.3%
IL-4–/IFN-γ–, %	99.22 ± 0.19	93.12 ± 0.91	93.14 ± 1.79
IL-4+/IFN-γ–, %	0.66 ± 0.20*	6.78 ± 0.93	6.71 ± 1.75
IL-4–/IFN-γ+, %	0.02 ± 0.02	0.01 ± 0.01	0.03 ± 0.02
IL-4+/IFN-γ+, %	0.09 ± 0.05	0.09 ± 0.02	0.11 ± 0.04

LNs were isolated from mice on day 17. LNs of one group were collected as one population of LN cells. LN cells were stimulated with *Dp* (10 μg/ml) for 19 h. Then, cells were harvested and stained with FITC-conjugated anti-mouse IFN-γ mAb and PE-conjugated anti-mouse IL-4 mAb, and analyzed by FACScan. Data are presented as mean ± SE of three individual experiments. *$p < 0.01$ compared with the placebo group.

Dp-injected, FK506-treated mice was comparable to that of *Dp*-injected, placebo-treated mice. The cell number in the spleen was not different amongst the groups (data not shown).

Next, we analyzed freshly isolated LN by flow cytometry and compared the cell populations (table 1). The CD69+ population in *Dp*-injected, placebo-treated mice was enhanced compared to that in sham-injected mice. An analogous enhancement was observed in *Dp*-injected, FK506-treated mice. The absolute number of TCRαβ+ cells from both *Dp*-injected, placebo-treated mice and *Dp*-injected, FK506-treated mice was increased due to the increase in LN cells (fig. 6, table 1).

Topical application of FK506 ointment did not diminish either the cell expansion or cell activation in LNs. Next, we analyzed the Th1 to Th2 ratio in LNs and compared *Dp*-injected, FK506-treated mice with *Dp*-injected, placebo-treated mice (table 2). Regional LN cells were incubated with *Dp* for 19 h, and intracellular IL-4 and IFN-γ were evaluated by flow cytometric analysis. The *Dp*-specific IL-4+/IFN-γ– Th2 population in *Dp*-injected, placebo-treated mice (6.78 ± 0.93%) was greater than that in sham-injected mice (0.66 ± 0.2%). The *Dp*-specific Th2 population in *Dp*-injected, FK506-treated mice (6.71 ± 1.75%) was equivalent to that in *Dp*-injected, placebo-treated mice. The *Dp*-specific IL-4–/IFN-γ+ Th1 populations in LN cells of *Dp*-injected, placebo-treated mice and *Dp*-injected, FK506-treated mice were not different compared to those in sham-injected mice (table 2).

Effect of FK506 on the Untreated Ear

Though FK506 ointment did not modulate cell expansion, cell population or the Th1 to Th2 ratio in drainage LNs, it was effective on clinical signs and histopathological changes of the skin in this model. We hypothesized that FK506 ointment acts locally and does not exhibit a systemic effect by diffusion into the blood. To determine the local action of FK506 ointment, we examined skin

lesions in *Dp*-injected mice treated with FK506 on the contralateral ear. The thickness of the *Dp*-injected ear of mice treated with FK506 on the contralateral ear was similar to that in *Dp*-injected, placebo-treated mice (fig. 7). Clinical signs and histopathology of *Dp*-injected ears of mice treated with FK506 on the contralateral ear developed similarly to those in *Dp*-injected, placebo-treated mice.

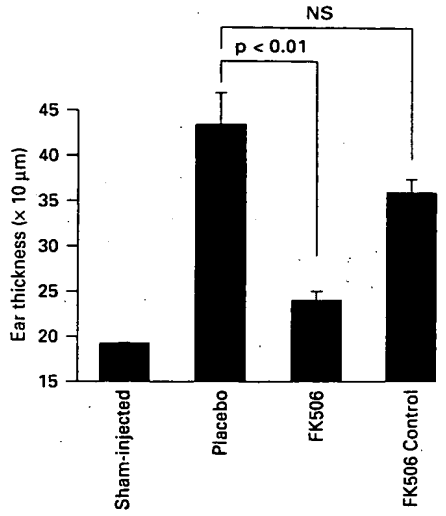


Fig. 7. Application of FK506 ointment to the contralateral ear did not affect the thickness of the Ag-treated ear. NC/Nga mice were treated and evaluated on day 17 as described in the legend to figure 1. Sham-injected mice, *Dp*-injected, placebo-treated (placebo) mice, *Dp*-injected, FK506 0.3% ointment-treated (FK506) mice and *Dp*-injected mice with FK506 0.3% ointment on the contralateral ear (FK506 Cont.) were investigated. Drugs were applied 3 h before and after each *Dp* injection. Each data point represents the mean ± SE of 10 mice. These data are representative of three separate experiments. NS = Not significant.

Discussion

In this study, we demonstrated that FK506 ointment as well as steroid treatment were effective in our newly developed mite Ag-induced dermatitis model in NC/Nga mice [5]. FK506 ointment reduced erythema, edema, scaling and excoriation. The effect of FK506 ointment could be exerted by topical mechanisms without any systemic immune suppression.

Histopathological study showed that FK506 ointment reduced the infiltration of CD4+ T cells and secretion of IL-4 and IFN-γ in the dermis of NC/Nga mice with mite Ag-induced dermatitis. CD4, IL-4 and IFN-γ were detected in the lesional skin of this model. However, our previous study showed that there was only a small population of IFN-γ+ cells in LNs [5]. In AD patients, IFN-γ has also been detected in severe eczema [13], but decreased production of IFN-γ from peripheral blood mononuclear cells has been observed [14]. IFN-γ in the skin closely correlates to delayed-type hypersensitivity, which exacerbates AD symptoms. However, systemic treatment with IFN-γ decreases the dermatitis of NC/Nga mice [15]. Thus, IFN-γ may have different roles in the local skin and the systemic immune system. It is possible that the suppression of local IFN-γ together with IL-4 by FK506 treatment may contribute to improvement of dermatitis. FK506 ointment also decreased CD4+ T cells in the local skin, suggesting that FK506 ointment inhibits both the infiltration and activation of CD4+ T cells in this model.

FK506 ointment reduced the infiltration of eosinophils and expression of ICAM-1 and VCAM-1, important adhesion molecules for eosinophil infiltration, in the dermis. Expression of ICAM-1 and VCAM-1 increased in the lesional skin of this model, and also occurs in the injured skin of AD patients. Although TNF-α and IL-1β are potent inducers of ICAM-1 and VCAM-1 in endothelial cells in vitro, we could detect TNF-α in this model (fig. 5), but not IL-1β (data not shown). This suggests that TNF-α rather than IL-1β might be important for the expression of ICAM-1 and VCAM-1 in this model. Since FK506 ointment suppressed TNF-α production and the infiltration of CD4+ T cells (potent producers of TNF-α) in the dermis, the suppressive effect on the expression of adhesion molecules could be attributed to inhibition of cytokine production and/or T cell migration.

Application of FK506 ointment to the contralateral ear did not suppress the increase in ear thickness of the Ag-treated ear, suggesting that FK506 ointment exerted a localized effect on the area of application under our experimental conditions. It is unlikely that the effect of FK506 ointment applied to the ear was due to oral uptake of the ointment by licking, since oral administration of FK506 was only effective at more than 10 mg/kg (data not shown), about 7 times more than the content of FK506 in the applied ointment. More importantly, treatment with FK506 ointment did not affect regional LN cells. Both CD69+ and IL-4+/IFN-γ+ Th2 populations in LNs increased after stimulation with *Dp*, and FK506 did not reduce this increase. These results indicate that topical application with FK506 ointment acts only locally under

our experimental conditions. It has been reported that topical FK506 decreases the expression of FcεRI and CD36 on CD1a+ epidermal dendritic cells in AD patients [16], indicating that topical FK506 treatment lowers the level of Ag uptake by dendritic cells. This suggests that topical FK506 treatment may modulate the immune response in LNs in clinical settings, since dendritic cells taking up Ag migrate into LNs and control the immune response. We did not detect any significant effect of FK506 ointment on LN cells. Since we injected rather a large amount of Ag into the skin to establish the model, this may mask the effect of FK506 on dendritic cells.

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